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**DEVELOPMENT OF A BIOPROCESS FOR PRODUCTION OF A
NEW *A. NIGER* FS3 PHYTASE: STUDIES OF ITS PURIFICATION AND
CHARACTERIZATION**

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obtaining the Doctor degree in
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“Men are not prisoners of fate, but only prisoners of their own minds.”

Franklin D. Roosevelt

“The important thing is never to stop questioning.”

Albert Einstein

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ABSTRACT

Phytases have important applications in human and animal nutrition because they hydrolyze the phytate present in legumes, cereal grains and oil seeds. This results in an increased availability of minerals, trace elements and amino acids as well as phosphate. Fifty potential phytase-producing fungal strains were isolated from a fertile soil obtained from the northern part of Paraná State in Brazil and other alternative sources using a selective media. Heat treatment resulted in an increase of inorganic phosphate concentration, which is well known as a microbial phytase production repressor, and also reduces phytase production. UV exposure of the substrate was shown to reduce microbial contamination without affecting phytase production.

A Plackett-Burman screening design was applied to identify significant physico-chemical variables in phytase formation. These pre-selected variables were subsequently optimized using a Central Composite Rotational Design (CCRD). The maximum phytase production was achieved with the optimum variables temperature 30°C, initial moisture 65%, Na-citrate buffer concentration 0.3M, initial pH 5.0 and urea concentration 1.5%. An overall 4.3-fold improvement in phytase production was successfully achieved.

Respirometric data of phytase production by citric pulp fermentation in a column-type bioreactor was monitored using a new data acquisition system. Respirometric activity during fermentation and its relation with fungal growth, forced air and enzyme production using a column-type bioreactor were also objectives of this work. Phytase synthesis by a new isolate, *A. niger* FS3, increased with forced air. The O₂ consumption and CO₂ production during solid-state fermentation were monitored by controllers, acquired by sensors in the bottom and top of the columns linked to controllers, recorded by the acquisition software and processed by Fersol2 software tool without the need of a gas chromatography. Phytase synthesis was associated to fungal growth, and therefore could be used to estimate biomass formed in citric pulp fermentation.

The identification of suitable conditions for the first step of phytase purification was performed in batch. The results showed that parameters established as SP-sepharose FF in glycine-HCl pH 2.85 and enzyme diluted in the same buffer in the

proportion 1:3 (v/v), resulted in a substantial recovery of enzyme (59.61%) compared to the recovery percentages in CM and DEAE-sepharose (56.61% and 37.64%, respectively) considering the study conducted in tubes instead of column. This batch study is a fast way to define parameters to run ion exchange chromatography. The phytase was purified to electrophoretic homogeneity by cationic-exchange, anionic-exchange chromatography and cromatofocusing steps. On SDS-PAGE analysis, the molecular weight of the purified phytase was calculated to be approximately 100 kDa. The phytase has an optimum pH of 5.0-5.5 and an optimum temperature of 60°C. The phytase displayed high affinity for phytate and the K_m was 0.52 mM.

INTRODUCTION

Phytases are a group of enzymes that presents a great importance in food and feed, although in Brazil there is still no commercial product for application in food processing. In Brazil the high volume of application of phytases is limited to feed. Thus the production by a Brazilian company, would lead to a reduction in its cost, which would meet the demand with respect to this important technological coadjuvant input. Moreover, Brazil has a rich biodiversity where find micro producers of this enzyme in the whole Brazilian territory, since these microorganisms can be isolated from the soil itself besides other sources. In addition to microbial biodiversity, there is also an abundance of agro industrial wastes that could be used as natural substrates for the production of phytase, which often have not of economical importance.

The importance of esters of inositol phosphates generated by phytate degradation is due to the benefits that these substances bring to human and animal health. At the same time that these substances are generated, phytates are degraded eliminating its anti nutritional effect.

The inositol phosphates have importance in the treatment of various diseases in humans. Among the main features are: reduction of heart disease, carcinogenic action, prevention of complications of diabetes, cardiovascular diseases and treatment of chronic inflammation.

In addition to the degradation the presence of antinutrients in plant sources, the importance to obtain inorganic phosphorus and its recovery is also another focus of interest to produce phytase, considering that phosphorus is a mineral scarce in Brazil. Brazil, with only 0.4% of world reserves, depends on imports. However, the world reserves of phosphate, essential for agriculture, may be exhausted within the next 60 to 100 years if it is maintained the current increment in worldwide consumption. Without this mineral, agriculture and even the production of fuel will be compromised.

The use of procedures for the separation of biomolecules such as enzymes requires specific equipment, reagents and procedures. Moreover, it is usually required a combination of methods to obtain the substance of interest highly pure.

OBJECTIVES

General purpose

This research work has as main goal to produce of a phytase synthesized by a fungus isolated from soil, using wastes from Brazilian agroindustry.

Specific purposes

- Select and isolate potential phytase-producing microorganisms from soil samples;
- Select agroindustrial wastes for use as substrate in fermentation process;
- Study the ideal conditions for phytases production, optimizing physical and chemical conditions of cultivation;
- Analyse the respirometric activity during fermentation and its relation with fungal growth, forced air and enzyme production using a column-type bioreactor;
- Identify suitable conditions in batch for the first step of phytase purification, and then purify using chromatography steps.
- Characterize the pure enzyme;

REVIEW

Phytic acid

Myo-inositol hexaphosphate (IP6, phytic acid) and its salts (phytates) are a natural compound frequently occurring in nature and constitutes the principal storage form of phosphorus and inositol in plants seeds and grains. In cereals and legumes, it represents 18-88% of total phosphorus content (Reddy et al 1982). During maturation of the plant seed and in dormant seeds, phytate is formed and it represents 60–90 % of the total phosphate (Loewus 2002). Phytic acid is a simple ringed carbohydrate with six phosphate groups attached to each carbon (Shamsuddin et al 2002), with a molecular formula $C_6H_{18}O_{24}P_6$ and a molecular weight of 659.86 (Vohra and Satyanarayana 2003). The unique structure, with 12 replaceable protons and high density of negatively charged phosphate groups, is responsible for its characteristic properties, allowing to form very stable complexes with multivalent cations (Dost and Tokul 2006). Salts of phytic acid are called phytates.

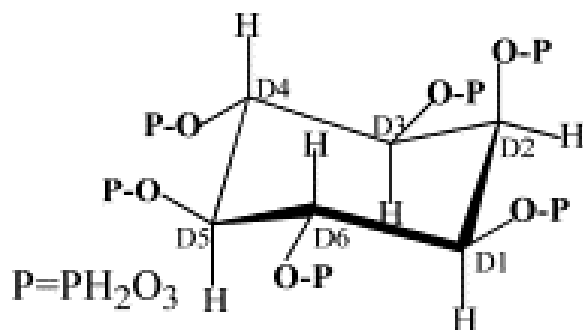


Fig. 1 – Structure of phytic acid (Source: Raboy 2003)

Phytate is therefore a common constituent of plant-derived foods (**Table 1**). Depending on the amount of plant-derived foods in the human diet and the grade of food processing, the daily intake of phytate can be as high as 4500 mg (Reddy 2002). On average, daily intake of phytate was estimated to be 2000–2600 mg for vegetarian diets as well as diets of inhabitants of rural areas in developing countries and 150–1400 mg for mixed diets (Reddy 2002).

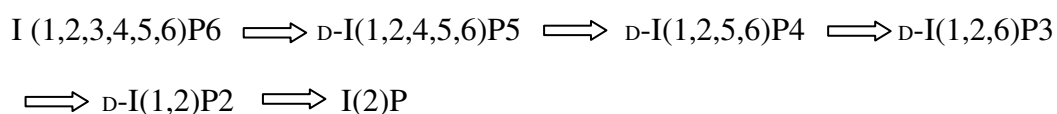
Table 1 - Phytate content per mass of dry matter in plant-derived foods

Food	Phytate (mg/g)	Food	Phytate (mg/g)
<i>Cereal-based</i>		<i>Legume-based</i>	
french bread	0.2-0.4	chickpea (cooked)	2.9-11.7
mixed flour bread (70% wheat, 30% rye)	0.4-1.1	cowpea (cooked)	3.9-13.2
mixed flour bread (70% rye, 30% wheat)	0-0.4	black beans (cooked)	8.5-17.3
sourdough rye bread	0.1-0.3	white beans (cooked)	9.6-13.9
whole wheat bread	3.2-7.3	lima beans (cooked)	4.1-12.7
whole rye bread	1.9-4.3	faba beans (cooked)	8.2-14.2
unleavened wheat bread	10.6-3.2	kidney beans (cooked)	8.3-13.4
maize bread	4.3-8.2	navy beans (cooked)	6.9-12.3
unleavened maize bread	12.2-19.3	Soybeans	9.2-16.7
oat bran	7.3-2.1	Tempeh	4.5-10.7
oat flakes	8.4-12.1	Tofu	8.9-17.8
oat porridge	6.9-10.2	lentils (cooked)	2.1-10.1
pasta	0.7-9.1	green peas (cooked)	1.8-11.5
maize	9.8-21.3	Peanuts	9.2-19.7
cornflakes	0.4-1.5	<i>Others</i>	
rice (polished, cooked)	1.2-3.7	Sesame seeds (toasted)	39.3-57.2
wild rice (cooked)	12.7-21.6	soy protein isolate	2.4-13.1
sorghum	5.9-11.8	soy protein concentrate	11.2-23.4
		Buckwheat	9.2-16.2
		amaranth grain	10.6-15.1

Source: Greiner 2006

Phytic acid acts as an antinutrient because of its ability to chelate essential minerals and proteins altering their solubility, functionality, digestibility and absorption (Rickard and Thompson 1997) and thus poses a nutritional problem in human and animals. Phytate behaves in a broad pH range as a highly negatively charged ion and has therefore a tremendous affinity for food components with positive charge(s), such as minerals, trace elements and proteins (Konietzny and Greiner 2003, Cheryan 1980). This interaction does not have only nutritional consequences, but also affects yield and quality of food ingredients such as starch; corn steep liquor or plant protein isolates (Wang 1999, Fredrikson 2001, Caransa 1988, Antrim 1997, Kvist 2005). The major concern about the presence of phytate in the human diet is its negative effect on mineral uptake. Minerals of concern in this regard include zinc, iron, calcium, magnesium, manganese and copper (Konietzny and Greiner 2003, Lopez 2002). The formation of insoluble mineral-phytate complexes at physiological pH values is regarded as the major reason for the poor mineral bioavailability, because these complexes are essentially nonabsorbable from the human gastrointestinal tract.

A simple representation of phytate degradation pathways by *Aspergillus ficuum* (Chen and Li 2003) is shown below:



Phytases

Phytases or phytate-degrading enzymes belong to a special class of phosphomonoesterases termed myo-inositol hexakisphosphate phosphohydrolases, which are capable of initiating the stepwise release of phosphate residues from phytate (salts of myo-inositol hexakisphosphate) (Greiner 2007).

Based on the amino acid residue in the active site, phytate-degrading enzymes can be referred to as histidine acid phosphatases, β -propeller phosphatases, cysteine phosphatases and purple acid phosphatases (Mullaney and Ullah 2007). Two classes of phytases are recognized by the International Union of Pure and Applied Chemistry and the International Union of Biochemistry (IUPAC-IUB): 3-phytase (EC 3.1.3.8) which initially removes phosphate from the D-3 position of the myo-inositol ring, and 6-phytase (EC 3.1.3.26), which preferentially initiates phytate dephosphorylation at the L-6 (D-4) position. However, phytate-degrading enzymes initiating phytate degradation at the D-5 and D-6 positions, respectively, have been found in nature (Barrientos et al 1994, Greiner et al 2000). Phytate-degrading enzymes from microorganisms are considered to be 3-phytases, whereas 6-phytases are said to be characteristic of the seeds of higher plants.

The phosphate residues of phytate are released by phytate-degrading enzymes at different rates and in different order. Independent of their bacterial, fungal or plant origin, the majority of the phytate-degrading enzymes exhibiting an optimum for phytate hydrolysis under acidic pH conditions release five of the six phosphate residues of phytate, and the final degradation product was identified as myo-inositol 2-phosphate (Konietzny and Greiner 2002).

Regulation of phytases formation

In mould, phytases formation is growth-associated (Vats and Banerjee, 2002, Greiner 2007). Enzyme starts to increase from the beginning of growth and continues to increase up to the onset of the stationary phase. Among the nutrient limitations tested, only carbon starvation was able to provoke an immediate synthesis of a phytate-degrading enzyme in *R. terrigena*. A tight regulatory inhibition of the formation of phytases by phosphate levels was generally observed in all microorganisms, including moulds, yeast and bacteria, but only rarely could microorganisms utilize phytate as the sole source of carbon and phosphate. Phosphate was shown to exert repression of phytase synthesis at the level of transcription (Greiner 2007). Phosphate present in different ingredients inhibited phytase synthesis in several species of yeasts and mold (Shieh and Ware 1968, Yamada et al 1968, Nayini and Markakis 1984)

Expression of phytases also depends on the nature of carbon source, initial pH and temperature used to cultivate the microorganisms. Both biomass and phytase production respond to all these parameters, but temperature and pH for maximal biomass and phytase production were shown to be different in some moulds and yeasts (Andlid et al 2004, Kim et al 1999, Sano et al 1999, Greiner 2007).

In the presence of simple sugars a strong repression of phytase formation was observed in several microorganisms, including bacteria, yeasts and moulds (Shieh and Ware 1968, Han and Gallagher 1987, Sano et al 1999, Lan et al 2002, Vats and Banerjee 2002, Han and Gallagher 1987, Lambrechts et al 1993).

In moulds such as *Aspergillus niger*, formation of mycelia pellets in the presence of glucose or fructose as the sole carbon source was shown to be responsible for the low enzyme yields (Shieh and Ware 1968, Han and Gallagher 1987). Dispersed growth and therefore an increase in phytase production could be obtained by using a medium containing a surfactant (Greiner 2007).

Some residues such as wheat and rice bran are excellent substrates for extracellular phytase production by microorganisms. As phytic acid is less soluble than sodium phytate, phosphate concentrations are lower due to a slower release from bran phytate, and therefore repression of enzyme synthesis by phosphate is reduced

(Greiner 2007). This ensures a continuous production of phytate-degrading enzymes during the whole fermentation process.

Application of phytases in feed

During the last 30 years, research has led to increased use of soybean meal and other plant material as protein sources in animal feed. In plant meal, one factor that must to be overcome is the presence of phytate, an antinutritional factor. Phytate phosphorus is not digested by monogastric animals (e.g., hogs and poultry), and in order to supply enough of this nutrient, additional phosphate is required in the feed ration (Mullaney et al 2000). Phytases are also applied as feed additive for fishes.

Over the last decade, numerous feed studies have established the efficacy of a fungal phytase from *A. niger* NRRL 3135, in hydrolyzing phytin phosphorus in an animal's digestive tract, which benefits the animal while reducing total phosphorus levels in manure (Mullaney et al 2000). The phosphorus thus released is transported into the water bodies causing eutrophication (Bali and Satyanarayana 2001). This results in oxygen depletion due to excessive algal growth. It also may affect the amount oxygen in water, leading to death of fish, hypoxia (Mallin 2000). The enzyme minimizes the need for supplementation with inorganic phosphorus due to improvement in the utilization of organic phosphorus in poultry, and this markedly reducing the excretion of phosphorus in manure (Mohanna and Nys 1999). Phytase hydrolyzes phytate, and the addition of phytase to feed (250 to 1000 U/kg) can fully replace phosphorus supplementation (Golovan et al 2001).

Application of phytases in food processing

There is also a great potential for the use of phytases in food processing and manufacturing for human consumption, but up to now, no phytase product for a relevant food application has found its way to the market. Research in this field focuses on the improvement of the nutritional value of plant-based foods as well as on the technical improvement of food processing. A diet rich in phytate leads to a considerably reduced absorption of dietary minerals (Konietzny and Greiner 2003, Lopez et al 2002) and the dephosphorylation of phytate during food processing results in the formation of only partially phosphorylated myo-inositol phosphate esters with a lower capability to impair the intestinal uptake of dietary minerals (Sandberg et al 1999, Sandström and Sandberg 1992, Han et al 1994). Individual myo-inositol

phosphate esters have been shown to have several important physiological functions in man (Shears 1998). Therefore, phytases may find application in food processing for production of functional foods (Greiner 2002), if such biochemically active myo-inositol phosphate esters could be generated by phytases and absorbed in the alimentary tract of humans. Technical improvements by adding phytases during food processing have been reported for breadmaking (Haros and Rosell 2001), production of plant protein isolates (Wang et al 1999, Fredrikson et al 2001), corn wet milling (Caransa 1988, Antrim et al 1997) and the fractionation of cereal bran (Kvist et al 2005).

Benefits of phytase action

The antinutritional properties of phytates can be reduced through their enzymatic dephosphorylation into lower phosphorylated products (Reddy 2002). Furthermore, the generation of these intermediate products (IP₁₋₄) and myo-inositol could also have relevant health implications, as they are involved in the regulation of vital cellular functions (Shamsuddin 2002).

Phytases are particularly important in human nutrition for their possible role in the degradation of phytate during both food processing and gastrointestinal transit (Sandberg and Andlid 2002). Previous studies have demonstrated that the degradation of phytate in the stomach and intestine is mainly due to dietary phytases and, probably, to the metabolic activity of the colonic microflora (Sandberg and Andlid 2002, Wise and Gilbert 1982). Individual *myo*-inositol phosphate esters have been shown to have important physiological functions in man (Shears 1998). Some of these compounds, in particular D-*myo*-inositol(1,4,5)trisphosphate and D-*myo*-inositol(1,3,4,5)tetrakisphosphate, have been demonstrated to play an important role as intracellular second messengers (Shears 1998), and several isomers of *myo*-inositol phosphates have shown important pharmacological effects, such as prevention of diabetes complications and anti-inflammatory effects (Carrington et al 1993, Claxon et al 1990), as well as antiangiogenic and antitumour effects (Maffucci et al 2005). In addition, dietary *myo*-inositol phosphates have been suggested to bring about benefits for human health, such as amelioration of heart disease conditions by controlling hypercholesterolemia and atherosclerosis (Jariwalla et al 1990), prevention of renal

stone formation (Grases et al 2000), and protection against a variety of cancers, in particular colon cancer (Vucenik and Shamsuddin 2003).

Engineering of phytases

Because phytases with the required properties for food processing applications have not been found in nature so far, engineering of phytases in order to optimise their catalytic features is seen as a promising strategy. Enhancement of thermal tolerance and increase in specific activity are two important issues not only for animal feed, but also for food processing applications of phytases. Different strategies have been used to obtain enzymes capable of withstanding higher temperatures. A shift in temperature optimum of the *Escherichia coli* phytase from 55 °C to 65 °C and a significant enhancement in its thermal stability at 80 °C and 90 °C was achieved by expression of the enzyme in the yeast *Pichia pastoris* after introduction of three glycosylation sites into the amino acid sequence of the *Escherichia coli* phytase by site-directed mutagenesis (Rodriguez et al 2000). Gene site saturation mutagenesis technology was a further approach used to optimise the performance of the *Escherichia coli* phytase (Garrett et al 2004). A library of clones incorporating all 19 possible amino acid changes in the 431 residues of the sequence of the *Escherichia coli* phytase was generated and screened for mutants exhibiting improved thermal tolerance. The best mutant showed no loss of activity when exposed to 62 °C for 1 hour and 27 % of its initial activity after 10 minutes at 85 °C, which is a significant improvement over the parental phytase. In addition, a 3.5-fold enhancement in gastric stability was observed. By using the consensus approach, which is based on the comparison of amino acid sequences of homologous proteins and subsequent calculation of a consensus amino acid sequence using one of the available standard programmes, a fully synthetic phytase was generated, which exhibited a 21-42 °C increase in intrinsic thermal stability compared to the 19 parent fungal phytases used in its design (Lehmann et al 2002). Furthermore, a 3-fold increase in specific activity was achieved by replacing a single amino acid in the sequence of a fungal phytase by site-directed mutagenesis (Tomschy 2000a and 2000b).

Finally, a phytase will not be competitive if it cannot be produced in high yield and purity by a relatively inexpensive system. Therefore, highly efficient and cost-effective processes for phytase production by recombinant microorganisms have been

developed. High levels of phytate-degrading activity accumulating in the fermentation medium has been described by using economically competitive expression/secretion systems for *Escherichia coli* (Miksch et al 2002) as well as for the yeasts *Hansenula polymorpha* (Mayer et al 1999) and *Pichia pastoris* (Yao et al 1998).

In order to increase phytate-degrading activity during food processing, incorporation of plants with a high phytase activity into the plant-derived raw material to be processed is seen as an alternative. The seeds of rye, triticale, wheat and barely are naturally high in phytase activity. In addition, the introduction and expression of microbial phytase-encoding genes into several different plants including tobacco (Verwoerd et al 1995, Ullah et al 1999, Pen et al 1993, George et al 2005, Lung et al 2005, Yip et al 2003), alfalfa (Ullah et al 2002), arabidopsis (Lung et al 2005, Coello et al 2001, Richardson et al 2001, Mudge et al 2003) subterranean clover (George et al 2004), sesame (Jin et al 2004), soybean (Li et al 1997), canola (Ponstein et al 2002), potato (Ullah et al 2003), rice (Lucca et al 2001, Zimmermann et al 2003, Lucca et al 2001, Hamada et al 2005), wheat (Brinch-Pedersen et al 2000) and sugarcane (Santosa et al 2004) have been reported. The introduced microbial phytase-encoding gene is mainly derived from *Aspergillus niger*, but also *Bacillus subtilis*, *Aspergillus fumigatus*, *Escherichia coli*, *Schwanniomyces occidentalis*, and *Selenomonas ruminantium* were used as the gene source. Beside the proof of concept, two main objectives were followed; expression of the phytase in the plant seed and expression of a phytase secreted by the plant root. Phytase-expressing transgenic seeds were discussed as a novel feed additive for improved phosphorus utilisation in animal agriculture, because it was shown that only a limited amount of transgenic seed is required in compound feeds to ensure proper degradation of the phytate present in animal diets during digestion in the stomach (Pen et al 1993).

CHAPTER I

PHYTASE PRODUCTION USING CITRIC PULP AND OTHER RESIDUES OF THE AGRO-INDUSTRY IN SSF BY FUNGAL ISOLATES

Abstract

Phytases have important applications in human and animal nutrition because they hydrolyze the phytate present in legume, cereal grains and oil seeds. This results in an increased availability of minerals, trace elements and amino acids as well as phosphate. Fifty potential phytase-producing fungal strains were isolated from a fertile soil obtained from the northern region of Paraná State in Brazil and other alternative sources using a selective media. Thereafter phytase production was evaluated in solid-state fermentation using different residues from the agro industry supplemented with a nitrogen source at 60 % of moisture after 96 hours at 30 °C. The highest phytase activity (51.53 units per gram of dry substrate, U/gds) was achieved with citric pulp and the soil isolated FS3 in solid-state fermentation. Furthermore, treatment of the substrates prior to fermentation in order to reduce microbial contamination was shown to affect phytase production during solid-state fermentation. Heat treatment resulted in an increase of the concentration of inorganic phosphate, a well known repressor of microbial phytase production, and therefore in a reduction of phytase production. UV exposure of the substrate was shown to reduce microbial contamination without affecting phytase production.

Key words: phytase, fungal strains, isolation, solid-state fermentation, citric pulp, phytate, agroindustrial residues

1. Introduction

Citric pulp (CP) is a solid residue resulting from the processing of oranges. Products derived thereof include dehydrated feed, pectin for food processing, citric acid, essential oils, molasses, and candied peel (Nelson and Tressler 1980). Once the juice has been extracted from the oranges, peels and rags are left behind. They are equivalent to approximately 50 % of the processed fruit mass and are converted into CP pellets (Abecitrus 2007). This material, with a moisture level of about 12 % CP was suggested to be an economic alternative substrate for enzyme production by solid-state fermentation.

Phytates (*myo*-inositol 1,2,3,4,5,6-hexakisphosphate) are salts of phytic acid and the major form of phosphorus stored in seeds, fruits and legumes (Kim et al 1998, Wang, Swain and Hasseltine 1980). Considerable amounts of phytate can be found in plant-based food products such as rice bran, oat flour, barley flour, wheat bran, beans, sesame bran, sunflower meal, soybean, cowpea, and sorghum (Roopesh et al 2006, Dost and Tokul 2006, Lestienne et al 2005, Kaur and Satyanarayana 2005, Ebune et al 1995). Phytate is also the major form of phosphorus in soil (Turner et al 2002). It chelates vital ions, thereby reducing their solubility and bioavailability. Phytate also forms complexes with proteins, which might result in an inhibition of digestive enzymes. Thus, high intakes of phytate result in poor utilization of nutrients. Therefore, phytate is considered to be an anti-nutritional factor (Pallauf and Rimbach 1997, Nair and Duvnjak 1990, Erdman and Poneros 1989, Maga 1982).

Moreover, monogastric animals are unable to use phytate phosphorus, because they lack endogenous phytase activity in their digestive tracts (Wodzinski & Ullah 1996, Common 1989). To make the phytate phosphorus in the feed available to the animal and to reduce the addition of inorganic phosphorus to the feed in order to meet the animal's requirement for phosphorus, the use of phytases as feed supplement has steadily increased. Phytases (*myo*-inositol hexakisphosphate phosphohydrolases) are enzymes capable of initiating the hydrolysis of phytate resulting in partially phosphorylated *myo*-inositol phosphates and inorganic phosphate (Kim et al 1998, Jareonkitmongkol et al 1997, Nagai and Funahashi 1962).

The use of filamentous fungi for the phytase production through solid-state fermentation has gained much interest for research in the last years (Pandey et al

2001). The objective of this study is to compare phytase production in fungi isolated from soil and other habitats and evaluate the influence of different substrates, nitrogen source and citric pulp treatment for phytase production by the screened microorganisms.

2. Materials and Methods

2.1 Isolation of microorganisms

Soil, corn, CP pellets, wheat bran and rice bran were used as a source material to isolate fungi. One gram of each source material was suspended in 100 mL of 0.1 % (w/v) peptoned water. The suspensions were incubated aerobically at 120 rpm on a rotatory shaker at 30 °C for 24 hours. Then, 0.1 mL of a 10^{-2} to 10^{-8} dilution of the liquid medium in 0.1 % of peptoned water was plated onto a solid phytate-agar media containing (in g/L): phytate (Sigma) 10, NaNO_3 2, NH_4NO_3 2, $\text{MgSO}_4 \cdot 7 \text{H}_2\text{O}$ 0.5, KCl 0.5, $\text{ZnSO}_4 \cdot 7 \text{H}_2\text{O}$ 0.1, FeSO_4 0.05, agar 19 and gentamicin sulphate (Shering-Plough) as described by Vats and Banerjee (2002). The plates were incubated at 30 °C for 5 days. To isolate pure microorganisms, single colonies were transferred to potato dextrose agar (PDA) plates and the plates were incubated at 30 °C for 7 days. Thereafter, the plates were stored at 4 °C until use.

2.2 Inoculum preparation

Inoculum was prepared by suspending the spores present on the PDA agar plates in 20 mL of 0.1 % Tween 80. The number of spores was determined in a Neubauer counting chamber and the inoculum of 10^7 spores/g CP was used for SSF.

2.3 Study of different substrates in SSF

Different solid substrates namely citric pulp (CP), apple pulp *var. Gala* (APG), soy bran (SB), wheat bran (WB), rice bran (RB), citric pulp + apple pulp *var. Gala* (CP + APG), citric pulp + soy bran (CP + SB), citric pulp + wheat bran (CP + WB), citric pulp + rice bran (CP + RB), in ratio 1:1, were used in SSF in order to study their effect on phytase production by *Aspergillus* FS3. The fermentations were carried out in 250-mL Erlenmeyer flasks at 30 °C for 96 h with an initial moisture content of 60 %, a pH value of 5.0 and ammonium citrate as nitrogen source.

2.4 Phytase production in SSF

Fermentation was carried out in 250-mL Erlenmeyer flasks containing CP as the solid substrate and ammonium citrate as nitrogen source. The moisture level was adjusted to 60 % with ultra pure water. Cotton-plugged flasks were incubated at 80 °C for 24 h followed by exposure to UV light for 5 h. After cooling down to room temperature, the flasks were inoculated with 10^7 spores/g of CP of the corresponding microorganism. The contents of the flasks thoroughly mixed, then incubated at 30 °C for 96 h, after which the content were harvested and assayed for phytase activity. All experiments were done in triplicate.

2.5 Pre treatment of CP

The effect of different pre treatments of CP for phytase production was studied to reduce microbial contamination (Table 1). The experiments were conducted in 250 mL Erlenmeyer flasks containing CP (Cargill SA, Brazil) as the substrate in SSF. The moisture level was adjusted to 60 % with citrate buffer, pH=5.0. After the pre treatment, the flasks were cooled down to room temperature and inoculated with the soil isolate FS3 and *A. ficuum* NRRL 3135. The contents of the flasks were thoroughly mixed and the flasks were incubated at 30 °C for 96 h. The contents of the flasks were harvested and assayed for phytase activity. All experiments were performed in triplicate.

Table 1 – Thermal pre treatment conditions of CP

Thermal pre treatment of CP	Conditions
Control	no thermal treatment, exposure to UV for 2 h
Autoclave (AS1)	dry (moisture 12 %), 121 °C for 15 min
Autoclave (AS2)	dry (moisture 12 %), 121 °C for 15 min
Autoclave (AU1)	wet (moisture 60 %), 121 °C for 15 min
Autoclave (AU2)	wet (moisture 60 %), 121 °C for 15 min
Fluent steam (VF20)	wet (moisture 60 %), 100 °C for 20 min
Fluent steam (VF40)	wet (moisture 60 %), 100 °C for 40 min
Fluent steam (VF60)	wet (moisture 60 %), 100 °C for 60 min
Dry incubator (E4)	dry at 65 °C for 4 h, exposure to UV for 2 h
Dry incubator (E8)	dry at 65 °C for 8 h, exposure to UV for 2 h
Dry incubator (E12)	dry at 65 °C for 12 h, exposure to UV for 2 h
Dry incubator (E24)	dry at 65 °C for 24 h, exposure to UV for 2 h
Dry incubator (E48)	dry at 65 °C for 48 h, exposure to UV for 2 h

*AS1, AS2 (CP autoclaved without moisture adjustment); AU1, AU2 (CP adjusted to 60% of moisture before autoclaving).

2.6 Study of different nitrogen sources

After choosing the best substrate for phytase production it was evaluated the effect of 0.5 % (w/v) of different nitrogen sources (ammonium nitrate, ammonium citrate, sodium nitrate, ammonium sulphate, potassium nitrate, ammonium chloride) for phytase production in SSF.

2.7 Crude phytase extraction

Four gram of the fermented substrate was mixed with 40 mL of citrate buffer (pH 5). The extraction was performed at 4 °C for 15 min under agitation at 120 rpm. The extract was clarified by filtration and centrifugation at 4500 rpm for 15 min. The clear extracts were used in a suitable dilution for phytase activity determination.

2.8 Phytase assay

Phytase activity was determined by quantification of the phosphate released from phytate during the enzymatic reaction (Taussky and Schorr 1953). A volume of 1 mL of the clear extract was diluted 1:4 in acetate buffer (pH=5.0) and incubated at 50 °C for 10 min with 1 mL of 1.5 mM Na-phytate (Sigma) in 0.1M citrate buffer, pH=3.0. The reaction was stopped by adding 1 mL of 10 % (m/v) trichloroacetic acid solution (TCA). A blank sample was prepared by mixing 1 mL of sodium phytate solution, 1 mL of TCA solution and 1 mL of the diluted extract. After incubation, 1 mL of distilled water and 5 mL of Taussky-Schorr (TS) reagent as described by Harland and Harland (1980) were added to the blank samples and the enzymatic reactions. After 10 min at room temperature, absorbance at 660 nm was read. One unit (U) of phytase activity is defined as the amount of enzyme that liberates one μmol of inorganic phosphate per minute under assay conditions.

2.9 Inorganic phosphate determination

Spectrophotometric quantification of inorganic phosphate was performed using the Taussky-Schorr reagent (Taussky and Schorr 1953). The determinations were performed in triplicate.

3.0 Moisture content and water activity

Moisture content was analyzed using a moisture determinator (BEL Engineering Topray BR-001). Water activity (a_w) was determined using an AQUALAB CX-2.

3. Results and Discussion

3.1 Isolation and screening of phytase-producing microorganisms

Soil, CP, corn, rice bran and wheat bran were used as a source material to screen for phytase-producing microorganisms. The focus of the work was to isolate fungal species as producers of an extracellular phytase. The main criterion to identify such fungal species was a significant growth on a phytate agar plate. As phytate was the only source of carbon and phosphorus, fungal species capable of growing on such agar plates have to produce an extracellular phytase in order to use phytate carbon and

phosphorus. A total of 50 strains showed good growth on phytate agar: 25 strains isolated from soil (FS1 to FS25), 7 from CP pellets (FCP1 to FCP7), 6 from corn (FC1 to FCP6), 8 from rice bran (FRB1 to FRB6) and 4 from wheat bran (FWB1 to FWB4). Phytase production by all strains screened as phytase producers was investigated in solid-state fermentation using CP as a substrate. All strains with the exception of 8 also showed significant phytase production in SSF. With 32 U/gds, the soil isolate FS3 was the best phytase producer. The reference strain *A. ficuum* NRRL 3135 produced 26 U/gds phytase activity under the identical conditions in fermentation. Only 3 further soil isolates showed significant phytase production: FS16 (17.1 U/gds.), FS22 (10.6 U/gds) and FS4 (13.1 U/gds), whereas with 6 soil isolates no significant phytase activity was obtained. Strains isolated from CP, corn, rice bran and wheat bran showed relatively high phytase production. Out of 25 isolated strains, 12 produced more than 10 U/gds with CP as a solid substrate. The highest phytase production was achieved with FCP2, a CP isolate (12.7 U/gds) and FC5, a corn isolate (16.81 U/gds).

The course of phytase production of the 12 best phytase producers was studied in SSF at 30 °C using CP as a solid substrate within 24 hours (Fig. 1). During the adaptation phase (within the first 24 hours of fermentation) no significant phytase production was observed. Thereafter, all strains began to produce significant phytase activity. Under the conditions used, maximum phytase activity was achieved after about 96 hours of fermentation. Extending the fermentation resulted in a slight decrease in phytase activity, which might be due to proteolytic degradation of the enzyme. The soil isolate FS3, which was shown to be the best phytase producer, was chosen for studies to improve fermentation and enzyme extraction in order to achieve higher yields in phytase activity.

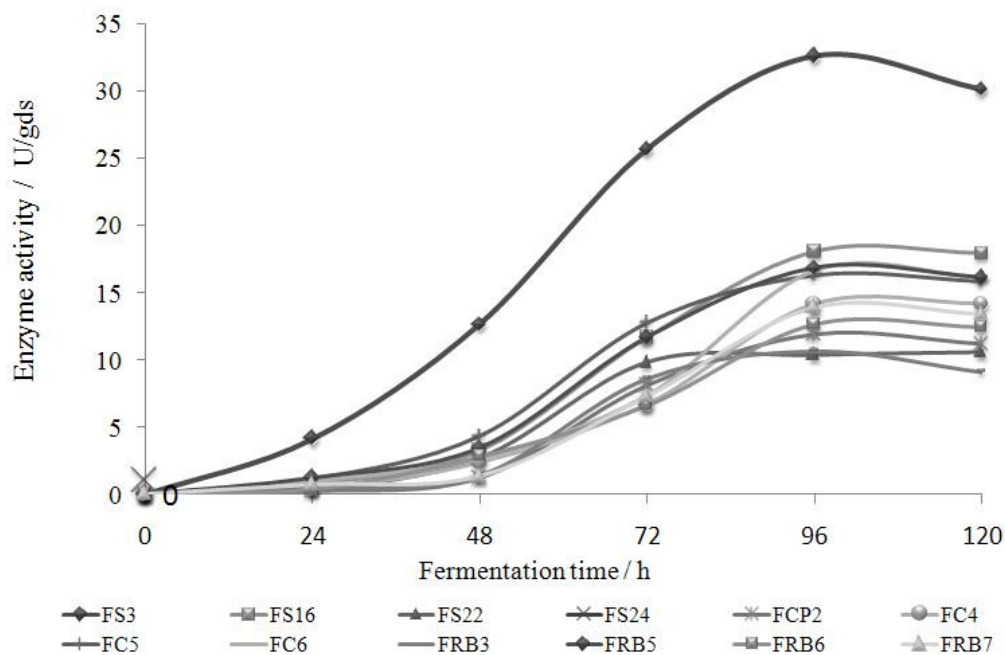


Fig. 1 - Profile of phytase production during SSF of citric pulp at 30 °C by different fungal isolates

3.2 Different substrates for phytase production by the isolate FS3

The energy required and the physical support for a fungus to grow and produce the desired metabolite(s) is primarily provided by the substrate (Pandey et al 2001). Thus besides CP, others agroindustrial residues were studied for phytase production during SSF (Fig. 2). All solid substrates investigated resulted in a significantly lower phytase production in comparison to CP, although substrates such as SB, WB and RB were commonly used for phytase production by SSF. Using WB as substrate, phytase activities have been achieved 9 U/gds by *Mucor racemosus* NRRL 1994 (Roopesh et al 2006) and 1.8 U/gds by *A. oryzae* AK9 (Chantasartasamee et al 2005). Using RB as a substrate, phytase activity of 15.8 U/gds was obtained with *A. oryzae* AK9 (Chantasartasamee et al 2005). Also, mixtures of the used substrates with CP did not enhance phytase titres when compared to CP itself. However, with the soil isolate FS3, the use of WB and RB as substrates resulted in the lowest phytase production. Fig. 2 clearly shows that phytase production during SSF is well correlated with the initial concentration of inorganic phosphate in the substrates used. Phosphate is a well known repressor of phytase synthesis by many fungi (Vats and Banerjee, 2002). Vohra

and Satyanarayana (2003) reported that maximum phytase production occurred in a medium containing only 5 µg/mL of inorganic phosphate, and higher concentrations resulted in a repression of phytase synthesis. This is in good agreement with the results reported here. Phytase production was higher in substrates with a low concentration of inorganic phosphate such as CP (0.025 µmol inorganic P/g), APG (0.028 µmol inorganic P/g) and CP+APG (0.011 µmol inorganic P/g), whereas SB, RB and WB contained higher amounts of inorganic phosphate.

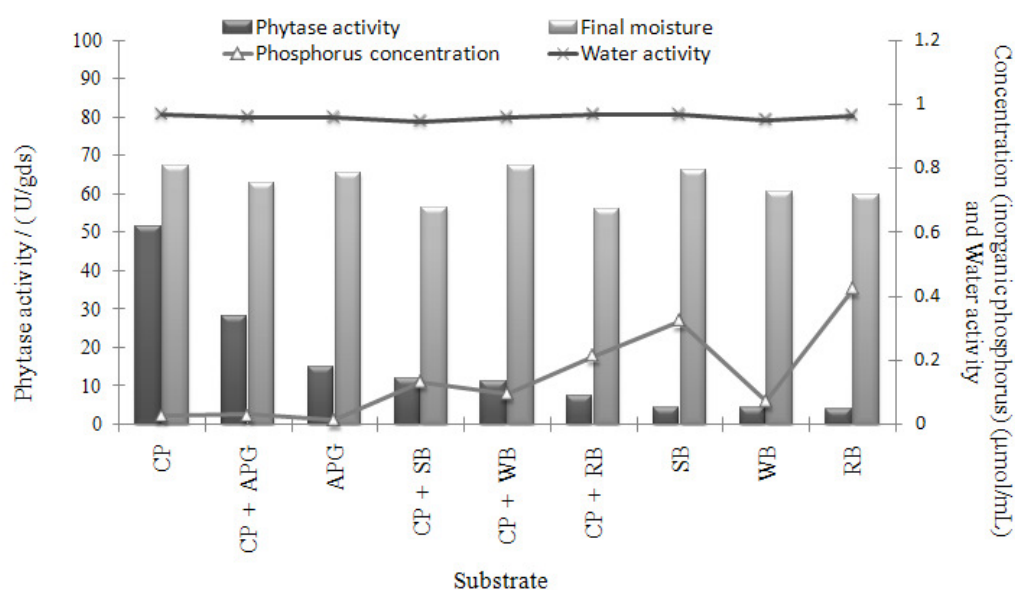


Fig. 2 - Phytase production during SSF using different agroindustrial residues by the soil isolate FS3

3.3 Effect of nitrogen source on phytase production

As indicated in Fig. 3, the nitrogen source used during SSF could greatly affect phytase production. Supplementation with a nitrogen source resulted in a better phytase production compared to the control without supplementation. Maximum phytase production was observed with both the control strain *A. ficuum* and the soil isolate FS3 when ammonium nitrate was used as a nitrogen source. This is in agreement with previous studies using *A. niger* van Tieghem as the fermentation organism (Vats and Banerjee 2002). Supplementation with ammonium citrate and

ammonium sulphate resulted also in a significant increase in phytase production compared to the unsupplemented controls.

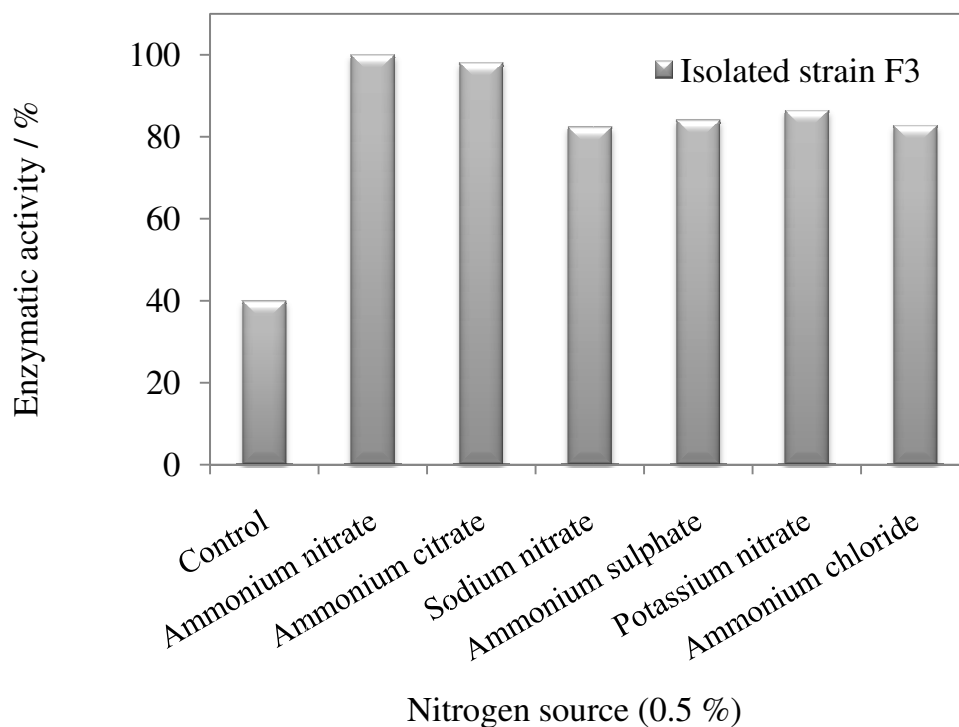


Fig. 3 - Effect of different nitrogen sources on phytase production by *A. ficuum* NRRL 3135 and soil isolate FS3 during SSF using CP as a solid substrate

3.4 Effect of CP thermal treatment on phytase production

Fig. 4 clearly shows that heat treatments of CP affects phytase production by the soil isolate FS3 during SSF. The best phytase production (44.83 U/gds) was observed after treating CP in a dry incubator for 4 h at 65 °C (E4). Longer drying times, higher drying temperatures, higher moisture content as well as autoclaving resulted in a significant lower phytase production during SSF. It could be suggested that these pre treatments resulted in a release of inorganic phosphate from phosphorylated compounds present in CP.

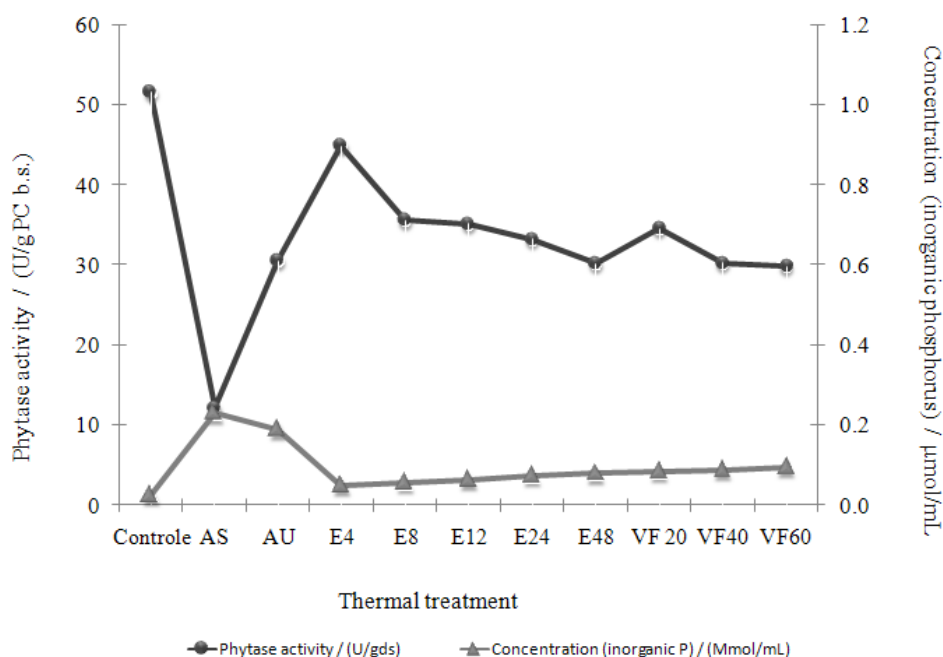


Fig. 4 - Effect of CP pre treatment on phytase production during SSF by FS3

4. Conclusions

Although a large number of fungi producing extracellular phytase could be isolated from natural habitats, only 12 strains (soil isolates: FS3 (32.2), FS16 (17.1), FS22 (10.6), FS24 (13.1), CP isolate: FCP2 (12.7), corn isolates: FC4 (14.7), FC5 (16.7), FC6 (16.8), rice bran isolates: FRB3 (10.9), FRB5 (16.9), FRB6 (12.5), (FRB7 13.9) achieved phytase activities higher than 10 U/gds in SSF using CP as a substrate. The soil isolate FS3 was identified as the best phytase producer. This strain was even better (32 U/gds) than the reference strain *A. ficuum* NRRL 3135 (26 U/gds). Although the substrates PG, SB, WB, RB also promote phytase synthesis by *A. FS3*, the yields were not as high as those obtained from CP fermentation. Combination of CP and the other substrates resulted in lower phytase production compared to CP itself. Thermal pre treatment of CP also resulted in a decreased phytase production. However, exposure to UV and a high inoculum rate have been proven sufficient to prevent growth of microbial contaminations during SSF.

References

- Nelson PE, Tressler DK (1980), Fruit and Vegetable Juice Processing Technology, AVI Publishing Co., Westport, USA pp. 603.
- Abecitrus (2007), Brazilian Association of Citric Products Exporters. Orange sub products, ABECitrus, Editorial Medrano, São Paulo, Brazil (<http://www.abecitrus.com.br>) (in Portuguese).
- Kim YO, Kim HK, Bae KS, Yu JH, Oh TK (1998), Purification and properties of thermostable phytase from *Bacillus* sp. DS11, *Enzyme Microb. Technol.* 22, 2-7.
- Wang HL, Swain EW, Hasseltine CW (1980), Phytase of molds used in oriental food fermentation, *J. Food Sci.* 45, 1264-1266.
- Roopesh K, Ramachandran S, Nampoothiri KM, Szakacs G, Pandey A (2006), Comparison of phytase production on wheat bran and oilcakes in solid-state fermentation by *Mucor racemosus*, *Bioresour. Technol.* 97, 506-511.
- Dost K, O. Tokul, Determination of phytic acid in wheat and wheat products by reverse phase high performance liquid chromatography, *Analytica Chim Acta*, 558 (2006) 22-27.
- Lestienne I, Icard-Vernière C, Mouquet C, Picq C, Trèche S (2005), Effects of soaking whole cereal and legume seeds on iron, zinc and phytate contents, *Food Chem.* 89, 421-425.
- Kaur P, Satyanarayana T (2005), Production of cell-bound phytase by *Pichia anomala* in an economical cane molasses medium: Optimization using statistical tools, *Process Biochem.* 40, 3095-3102.
- Ebune A, Al-Asheh S, Duvnjak Z (1995), Effects of phosphate, surfactants and glucose on phytase production and hydrolysis of phytic acid in canola meal by *Aspergillus ficuum* during solid-state fermentation, *Bioresour. Technol.* 54, 241-247.

Turner BL, Papházy MJ, Haygarth PM, McKelvie ID (2002), Inositol phosphates in the environment, *Phil. Trans. R. Soc.: Biol. Sci.* 357, 449–470.

Pallauf J, Rimbach G (1997), Nutritional significance of phytic acid and phytase, *Arch. Anim. Nutr.* 50,301–319.

Nair VC, Duvnjak Z (1990), Reduction of phytic acid content in canola meal by *Aspergillus ficuum* in solid-state fermentation process, *Appl. Microbiol. Biotechnol.* 34, 183–188.

Erdman LW, Poneros SA (1989), Phytic acid interaction with divalent cations in foods and in the gastrointestinal tract, *Adv. Exp. Med. Biol.* 249, 161-171.

Maga JA (1982), Phytate: Its chemistry, occurrence, food interactions, nutritional significance and methods of analysis, *J. Agric. Food Chem.* 30, 1-9.

Wodzinski RJ, Ullah AHJ (1996), Phytases, *Adv. Appl. Microbiol.* 42, 263-303.

Common FH (1989), Biological availability of phosphorus for pigs, *Nature*, 143, 370-380.

Jareonkitmongkol S, Ohya M, Watanabe R, Takagi H, Nakamori S, (1997), Partial purification of phytase from a soil isolate bacterium, *Klebsiella oxytoca* MO-3, *J. Ferment. Bioeng.* 83,393-394.

Nagai Y, Funahashi S (1962), Phytase (myo-inositol-hexaphosphate phosphohydrolase) from wheat bran. Part I. Purification and substrate specificity, *Agr. Biol. Chem.* 26, 794-803.

Pandey A, Szakacs G, Soccol CR, Rodriguez-Leon JA, Soccol VT (2001), Production, purification and properties of microbial phytases, *Bioresour. Technol.* 77, 203-214.

Vats P, Banerjee UC (2002), Studies on the production of phytase by a newly isolated strain of *Aspergillus niger* van Tieghem obtained from rotten wood-logs, *Process Biochem.* 38, 211-217.

Taussky HH, Schorr E (1953), A microcolorimetric method for the determination of inorganic phosphorus. *J. of Biol. Chem.* 202,675-685.

Harland BF, Harland J (1980), Fermentative reduction of phytic acid in rye, white and whole wheat bread, *Cereal Chem.* 57,226-229.

Pandey A, Szakacs G, Soccol CR, Rodríguez-Leon JA, Soccol VT (2001), Production, purification and properties of microbial phytases. *Bioresour. Technol.* 77,203-214.

Chantasartasamee K, Ayuthaya DI, Intarareugsorn S, Dharmsthiti S (2005), Phytase activity from *Aspergillus oryzae* AK9 cultivated on solid state soybean meal medium, *Process Biochem.* 40, 2285-2289.

Vohra A, Satyanarayana T (2003), Phytases: Microbial sources, production, purification and potential biotechnological applications, *Crit. Rev. in Biotechnol.* 23, 29-60.

CHAPTER II

OPTIMIZATION OF PHYTASE SYNTHESIS DURING CITRIC PULP FERMENTATION

Abstract

Citric pulp bran was used for the first time as substrate for production of phytase by a new isolated *A. niger* FS3 under SSF. A Plackett-Burman screening design was applied to identify significant physico-chemical variables. These pre-selected variables were subsequently optimized using a Central Composite Rotational Design (CCRD). The maximum phytase production was achieved with the optimum variables temperature 30°C, initial moisture 65%, Na-citrate buffer concentration 0.3M, initial pH 5.0 and urea concentration 1.5%. An overall 4.3-fold improvement in phytase production was successfully achieved.

Keywords: phytase, citric pulp bran, Plackett-Burman design, Central Composite Design, solid state fermentation

1. Introduction

In the last years, citric pulp bran has received increasing attention as one of the most important agro industrial residues in Brazil, from the orange producer chain and exporters of orange concentrated juice. Brazil is responsible for about 47% of the world orange juice production (IBGE, 2008). Citric pulp bran is a residue generated during the orange processing, consisting of bagasse, rags and seeds (IBGE, 2008; Abecitrus 2008, Nelson & Tressler 1980, Spier et al 2008, Citroex, 2008). The nutritional composition of citric pulp bran comprehends: proteins 5.5-6.2%; carbohydrates 70%; crude fiber 14%; lipids 1.2-1.3% and mineral matter < 8% (Abecitrus 2008, Citroex 2008). Among these nutrient compounds, low inorganic-P content (Oliveira et al 2004; Spier et al 2008) is one of characteristics that differ from others residues used for phytase production. Although citric pulp has low phosphate content, it presents other macro-elements such as calcium, magnesium, potassium and micro elements aluminum, barium, titanium, cooper, manganese, thorium, vanadium, zinc, lanthanum, samarium, cobalt, antimony, arsenic, and scandium (Oliveira et al 2008).

In the last few years, several studies have showed the use of sub-products of wheat, soy, oat, rice and other residues rich in phytate, but there is no data reporting the use of sources poor in phytate content and low inorganic-P sources to produce phytase as well as the optimization of production using citric pulp bran as substrate and support in solid state fermentation. Low inorganic-P substrate stimulates phytase synthesis (Spier et al 2008), but excess of inorganic-P causes a well known repression of phytase synthesis in many fungi (Vats Banerjee 2002); in concentrations above 5 $\mu\text{g/mL}$ for *Candida krusei* (Quan et al 2001) and above 0.5% for *A. niger* var teigham (Vats Banerjee 2002), above 0.2 mM in *A. niger* van Teighem (Vats and Banerjee 2006) and according to Kim et al (1998) and Bhavsar et al (2008), inorganic phosphorus concentration above 0.005% also showed adverse effect on phytase production. Phytase activity in *S. cerevisiae* was also repressed by inorganic phosphate in the medium (Andlid et al 2004), although the presence of traces of inorganic phosphorus is an essential ingredient of phytase production medium (Soni and Khire 2007) and induces its production. Phytase synthesis begins when the phosphate in the culture medium is consumed by the cells and the repression of

phytase synthesis by phosphate is eliminated (Quan et al 2001). Han et al (1987) found that 10 mg of phosphorus per 100 g of substrate in the growth medium was optimum for the enzyme production, while higher levels inhibited phytase production in SSF by *A. ficuum*. Citric pulp bran shows low inorganic-P content (0.025 μmol per gram of citric pulp) when compared to other agro industrial residues such as wheat bran, soy bran, rice bran and apple pulp already studied (Spier et al 2008). This characteristic may be an interesting reason to employ citric pulp bran, as a phytase inducer substrate.

Fermentation processes are significantly influenced by various physical and chemical parameters. The first step in process optimization is the screening of the important variables, followed by estimation of optimal levels of these factors (Li et al (2008). Since conventional methods or 'one variable at a time' approach are extremely time consuming, tedious and expensive for a large number of variables (Chadha et al 2004, Singh and Satyanarayana 2006, Li et al 2008). Therefore, before the definition of the factors to use in the optimization studies is important to select these important factors using tools as statistical experimental designs, Plackett-Burman, CCD and RSM (Li et al 2008, Singh and Satyanarayana, 2006).

The present work aims was to optimize phytase production in citric pulp bran fermentation using statistical tools, considering that this residue was not applied in biotechnology processes yet and it shows economic viability to produce biomolecules of commercial interest such as phytase.

2. Materials and methods

2.1 Fungal strain

A fungal strain which is a good phytase producer was isolated from soil samples collected at Parana State (Brazil) (Spier et al 2008). The isolated fungal strain was grown on PDA medium and kept at 4°C.

2.2 Study of inoculum media

Different inoculum media was studied to maximize phytase production in SSF medium before the optimizing the fermentation process. Potato dextrose agar (PDA), czapek medium, and citric pulp extract were used in this study with the follow variations (Table 1): Commercial PDA (A), czapek complete medium (20.0 g/l sucrose, 0.5 g/l NaNO₃, 1.0 g/l KH₂PO₄, 1.0 g/lMgSO₄, 0.6 g/l KCl, and 0.05 g/l FeSO₄; pH 6.7) (B), czapek medium without KH₂PO₄ (C), citric pulp extract supplemented with 0.5 g/l NaNO₃ and 1.0 g/l KH₂PO₄ (D) citric pulp extract supplemented with 0.5 g/l NaNO₃ (E). The N-source and P-source supplements in citric pulp extract were the same present in Czapek complete medium. Citric pulp extract was prepared with 1:10 (milled citric pulp bran: water), sterilized and diluted to 1:10 with ultra pure water. All media were adjusted to pH 5.0 according previous studies (data not shown).

Table 1 - Composition of inoculum media

Medium	Composition
A	Potato dextrose medium (commercial)
B	Czapek medium (commercial)
C	Czapek (commercial) - P source
D	Citric pulp extract + N source + P source
E	Citric pulp extract + N source – P source

2.3 SSF fermentation

1.5 mL of inoculum were transferred to 250 ml flasks containing 15g citric pulp bran with 70% (0.8-2.0 particles size) and 30% (>2.0 particles). The variables temperature of fermentation, initial moisture, initial pH, buffer concentration and urea concentration were tested in different levels according to the optimization tools. The fermentation time was defined as 96 hours (Spier et al 2008).

2.4 Enzyme assay

Phytase activity was determined measuring the amount of liberated inorganic phosphate based on Heinonen and Lahti (1981). A volume of 350 μ L of substrate solution (2.5mM sodium phytate from Sigma) in 0.2 M sodium acetate buffer, pH 4.5 and 50 of μ L enzyme were co-incubated at 50°C for 30 minutes. The reaction was stopped with the addition of 1.5 mL of AAM-solution, (10 mM ammonium molybdate, 5N H₂SO₄ and acetone in the proportion 1:1:2) and 100 μ L of citric acid. One unit of enzyme activity was defined as the amount of enzyme capable of releasing one μ mol of inorganic phosphate per minute under the defined reaction conditions.

2.5 Selection of the most suitable variables values according to the one-variable-at-a-time approach

Previous tests using one variable at a time were performed in order to find suitable intervals for each variable. Temperature, initial pH, initial moisture, nitrogen source and its concentration, buffer concentration were variables tested separately in different levels (Fig.1).

2.6 Selection of the most important variables

The purpose of the first optimization step was to identify which variable(s) of the solid state fermentation have a significant effect on phytase production. Based on Plackett-Burman (PB) factorial design, each variable was examined at two levels: -1 for low level and +1 (**Table 2**) for high level (Plackett and Burman, 1946). This screening was employed to determine which variables significantly affect phytase production by *A. niger* FS3 in citric pulp bran (**Table 3**). The variables used at the PB were are temperature of fermentation (A), initial moisture (B), initial pH (C), buffer concentration (D), surfactant (E) and urea concentration (F) and their respective

uncoded levels are presented in **Table 2**. D₁, D₂, D₃ and D₄ represents dummy variables.

Plackett–Burman experimental design is based on the first order model $Z = b_0 + \sum b_i x_i$ where Z represents the phytase production (response), b_0 is the model intercept, b_i is the linear coefficient (effect), and x_i is the level of the independent variable. This model does not describe interaction among factors and it is used to screen and evaluate the important factors that influence the response (Plackett and Burman, 1946; Lofty et al 2007).

Table 2 - Real values for the levels of the tested variables

Variable	-1	+1
A (temperature, °C)	28	32
B (initial moisture, %)	60	70
C (initial pH)	4.0	6.0
D (buffer, %)	0.0	0.3
E (surfactant, %)	0.0	0.1
F (urea concentration, %)	0.5	1.5

Table 3 - Selection of important variables by Plackett-Burman design - Two level screening (>6 factors)

Run	Variables										
	A*	B*	C*	D*	E	F*	G	D ₁	D ₂	D ₃	D ₄
1	+1	-1	+1	-1	-1	-1	+1	+1	+1	-1	+1
2	+1	+1	-1	+1	-1	-1	-1	+1	+1	+1	-1
3	-1	+1	+1	-1	+1	-1	-1	-1	+1	+1	+1
4	+1	-1	+1	+1	-1	+1	-1	-1	-1	+1	+1
5	+1	+1	-1	+1	+1	-1	+1	-1	-1	-1	+1
6	+1	+1	+1	-1	+1	+1	-1	+1	-1	-1	-1
7	-1	+1	+1	+1	-1	+1	+1	-1	+1	-1	-1
8	-1	-1	+1	+1	+1	-1	+1	+1	-1	+1	-1
9	-1	-1	-1	+1	+1	+1	-1	+1	+1	-1	+1
10	+1	-1	-1	-1	+1	+1	+1	-1	+1	+1	-1
11	-1	+1	-1	-1	-1	+1	+1	+1	-1	+1	+1
12	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1

*Indicates significant factors; D₁, D₂, D₃ and D₄ represents dummy variables.

2.7 Optimization of the selected variables

The five most significant factors [Temperature (A), Initial moisture (B), Initial pH (C), buffer concentration (D), urea (F)] were optimized using the response surface methodology known as Central Composite Rotational Design (CCDR) for enhancing phytase production. The variables were studied at 5 different levels (-2, -1, 0, +1, +2) (**Table 4**) and 30 experiments were carried out (**Table 5**).

Table 4 - Variables screened in Central Composite Rotational Design (CCRD)

Factors	Levels				
	-2	-1	0	1	+2
A - Temperature, °C	26	28	30	32	34
B – Initial moisture, %	55	60	65	70	75
D – Buffer conc., M	0.1	0.2	0.3	0.4	0.5
F – Urea conc., %	0.5	1.0	1.5	2.0	2.5

The statistical software package ‘STAT 5.0’ was employed to analyze the experimental data. All variables were taken at a central code value of zero. The response phytase activities (Z) were obtained after running the experiments combining variables according to the plan (**Table 5**). A multiple regression analysis of the data was carried out for obtaining an empirical model that relates the response measured to the independent variables. A second order polynomial equation for a five factor system is:

$$Y = \beta_0 + \beta_1A + \beta_2B + \beta_3C + \beta_4D + \beta_5F + \beta_{11}A^2 + \beta_{22}B^2 + \beta_{33}C^2 + \beta_{44}D^2 + \beta_{55}F^2 + \beta_{12}AB + \beta_{23}BC + \beta_{13}AC + \beta_{14}AD + \beta_{24}BD + \beta_{34}CD + \beta_{15}AF + \beta_{25}BF + \beta_{35}CF + \beta_{45}DF$$

Where Y is the predicted response, β_0 intercept, $\beta_1, \beta_2, \beta_3, \beta_4, \beta_5$ linear coefficients, $\beta_{11}, \beta_{22}, \beta_{33}, \beta_{44}, \beta_{55}$ squared coefficients, $\beta_{12} + \beta_{23} + \beta_{13} + \beta_{14} + \beta_{24} + \beta_{34} + \beta_{15} + \beta_{25} + \beta_{35} + \beta_{45}$ interaction coefficients and A, B, C, D, F, $A^2, B^2, C^2, D^2, F^2, AB, BC, AC, AD, BD, CD, AF, BF, CF, DF$ are the interactions among the levels of the independent variables.

Table 5 - Central Composite Rotational Design (CCRD) employed in phytase optimization

Run	Variable			
	Temperature (°C)	Initial Moisture (%)	Citrate- buffer (M)	Urea (%)
1	-1	-1	-1	1
2	-1	-1	1	-1
3	-1	-1	-1	-1
4	-1	-1	1	1
5	-1	1	-1	-1
6	-1	1	1	1
7	-1	1	-1	1
8	-1	1	1	-1
9	1	-1	-1	-1
10	1	-1	1	1
11	1	-1	-1	1
12	1	-1	1	-1
13	1	1	-1	1
14	1	1	1	-1
15	1	1	-1	-1
16	1	1	1	1
17	-2	0	0	0
18	2	0	0	0
19	0	-2	0	0
20	0	2	0	0
21	0	0	-2	0
22	0	0	2	0
23	0	0	0	-2
24	0	0	0	2
25	0	0	0	0
26	0	0	0	0
27	0	0	0	0
28	0	0	0	0
29	0	0	0	0
30	0	0	0	0

2.8 Validation of the experimental model

The statistical model was validated to phytase production under solid state fermentation in flasks, using conditions predicted by the model: 15g citric pulp bran, 1.5% urea, 0.3M Na-citrate buffer, pH 5.0, initial moisture 65% incubated at 30°C for 96 hours. Samples were submitted at the desired intervals and phytase activity was assayed as described in item 2.5.

2.9 Effect of salts in phytase production

A Plackett-Burman design was used for the second time to investigate the most significant salts in phytase production. The tested salts (KCl, CaCl₂, MgSO₄, NaNO₃, FeSO₄, MnSO₄, ZnSO₄, CuSO₄ and CoSO₄) were examined at two levels: -1 (absence of salt) and +1 (presence of salt), and the respective concentrations (in mM) are presented at **Table 6**.

Table 6 - Real values for the levels of the salts used in Plackett-Burman design

Variable (mM)	-1	+1
A (KCl)	0.0	1.0
B (CaCl ₂)	0.0	0.5
C (MgSO ₄)	0.0	1.0
E (NaNO ₃)	0.0	1.0
F (FeSO ₄)	0.0	0.5
G (MnSO ₄)	0.0	0.5
H (ZnSO ₄)	0.0	1.0
I (CuSO ₄)	0.0	0.5
J (CoSO ₄)	0.0	0.5

3. Results and Discussion

3.1 Study of inoculum medium type

Normally, the media used for inoculum preparation for fungi cultivation is PDA, Czapek and synthetic-based media. Several studies describe the use of these media to prepare inoculum for SSF for phytase production (Roopesh et al 2006, Chantasartrasamee et al 2005, Ramachandran et al 2005, Quan et al 2004). The present study showed an alternative for substitute the traditional synthetic media for fungi cultivation. The maximum phytase activity in SSF was achieved when inoculum medium was prepared with citric pulp supplemented with N-source and inorganic-P source (40.25 U/gds) (**Table 7**). The use of the same substrate used in fermentation processes to prepare inoculum medium demonstrated to be a cheaper alternative and showed best results in fermentation process to produce phytase. The possible reasons to explain the increase of phytase production were reduced adaptation period of microorganism in fermentation medium, due to the reproduction of the culture medium composition on fermentation composition. Certain types of residues cause dispersed mycelia growth and higher enzyme yield (Shieh and Ware, 1968), different of the growth obtained when only simple sugars are used as a sole source of carbon, where mycelial pellets were formed and low yields of phytase were observed in *A. ficuum* NRRL 3135 (Vohra and Satyanarayana, 2003).

A higher concentration of biomass was observed using citric pulp 0.84 and 0.754 gdm/L supplemented with P-source (D) and not supplemented (E), respectively (Table 4). The presence of P is fundamental to cell growth. According to Greiner (2007) a small amount of phosphate in the growth medium probably stimulates phytate-degrading enzyme formation by enhancing microbial growth. Similar effect occurred in SSF using the medium D, where citric pulp supplemented with inorganic-P-source stimulated biomass growth resulting in a higher phytase production comparing to the use of media A, B, C and E. In some cases, the residue does not have a complete nutritional requirement to microbial cell but may be supplemented with any desirable nutrient. So, in the case of inoculum D it was supplemented with nitrogen source and P-source, since citric pulp has low inorganic-P content as low protein content. Different nutrient media used for growing *A. niger* FS3 influenced the biomass formation, the number of pellets, residual inorganic-P and phytase activity in SSF.

The inoculum D was used in further studies of optimization because it showed maximum phytase production compared to inoculum media A, B, C and E.

Table 7 - Influence of inoculum media in biomass formation, number of pellets, residual inorganic-P and the posterior effect on phytase production in SSF

Inoculum media									SSF	
Cultivation media	Biomass (gdm/l)	Std deviation	Nº pellet	Std deviation	Pellet mass (mg)	Std deviation	Residual Inorganic-P	Std deviation	Phytase activity U/ml)	Std deviation
A	0.58	0.067	8	1	7250	2.45	0.42	0.03	30.42	1.87
B	0.57	0.044	7	1	8142.86	3.84	0.416	0.029	32.58	2.04
C	0.674	0.034	9	1	74888.89	2.08	0.243	0.043	35.67	2.84
D	0.84	0.057	13	2	6461.54	3.15	0.24	0.051	40.25	1.69
E	0.754	0.046	15	2	50266.67	4.91	0.287	0.049	37.84	2.34

3.2 Variables values definition

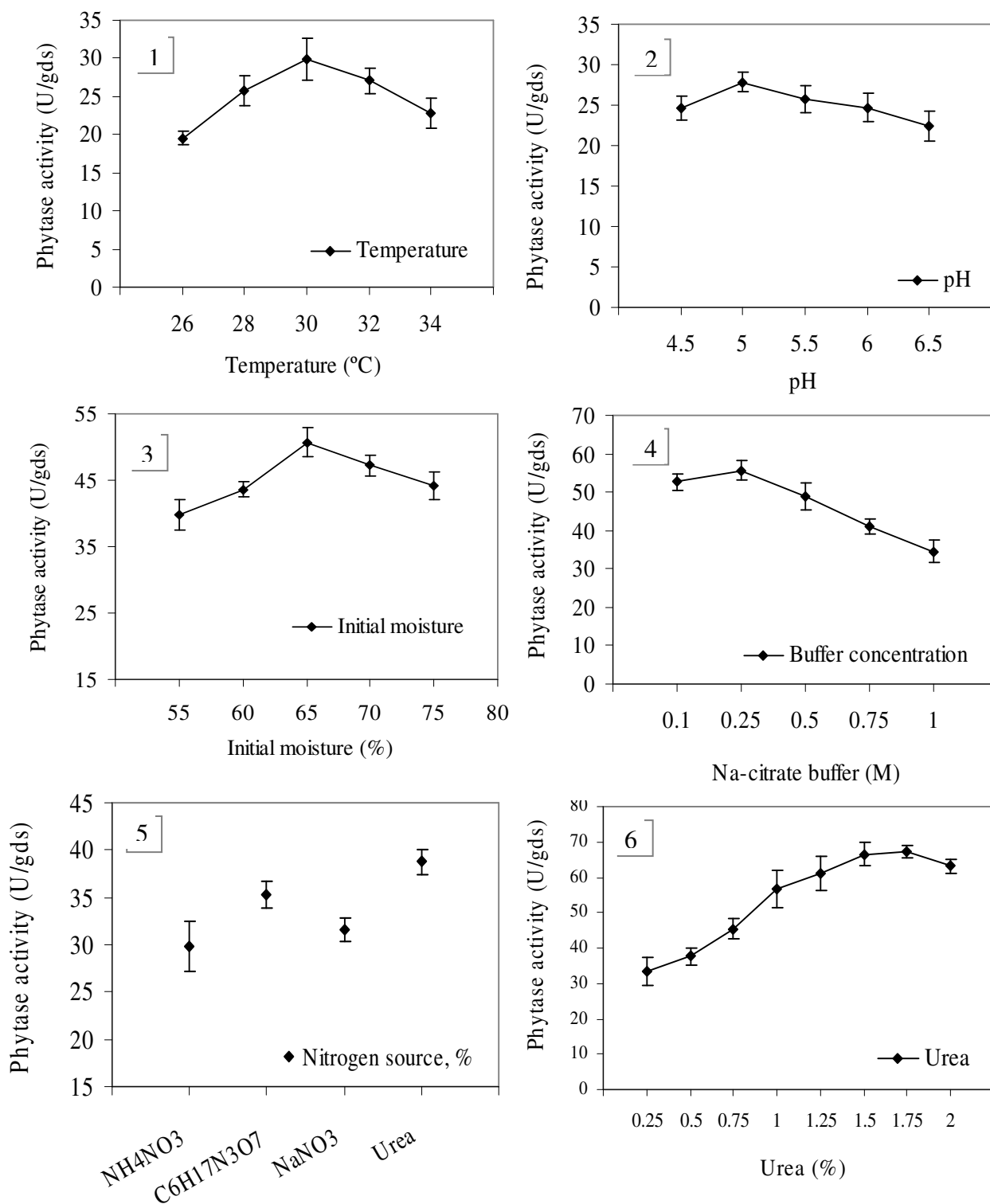


Fig. 1 – Previous studies in phytase production to define variables values in further experimental designs. 1) Influence of temperature; 2) Influence of pH; 3) Influence of initial moisture; 4) Influence of Na-citrate buffer; 5) Influence of different nitrogen sources; 6) Influence of urea.

According to results observed in Fig. 2 plots, the variables ranges for maximum phytase production tested separately were: temperature of fermentation 28-30°C (Fig.2.1), pH=4.5-5.5 (Fig.2.2), initial moisture of 60-70°C (Fig.2.3), Na-citrate buffer concentration (0.1-0.3M) and urea (1.25-2.0). The central level (0) of temperature of fermentation was defined at 30°C and it is in according to the previous studied and data in literature showing maximum enzyme activity found at 30°C (Vats and Banerjee 2002; El-Batal and Karem 2001). Previous studies in SSF were conducted to define the range of variables values adequate to phytase production.

3.2 Selection of important physico-chemical components

The main effects of the factors on phytase production are presented in **Table 10**. Values of “Prob>F” less than 0.05 indicates that model terms are significant at 95% of confidence level. The significative effects are (L for linear, Q for quadratic): temperature (A) L+Q; initial moisture (B) (Q); pH (C) (Q); buffer (D) (L+Q); urea (F) (L+Q), and the interaction between temperature (L) x buffer (L). These effects were used to estimate the maximum theorical response and the levels thereof.

Previous results of a screening using a Plackett-Burman with 7 variables and 12 experiments (**Table 3**), demonstrated that although the pH presented significative effect in response, did not show reasons to perform experiments outside of range -1 to +1 (4.5 to 5.5) and it was fixed in central point (pH=5.0) in accordance with results in Fig.2.2. For phytase production the optimum pH of most bacteria and fungi is in the range between 5.0 and 7.0 (Vohra and Satyanarayana 2003).

3.3 Optimization of the selected variables

A Composite Central Rotational Design (2^4 with 2 levels, 4 factors, with 8 axial points and 4 central points) was used to determine optimal levels of the selected variables that influenced phytase production. **Table 8** shows the results of the experimental plan for optimization of phytase production.

Table 8 - Composite Central Rotational Design (CCRD) for four variables: temperature of fermentation (°C), initial moisture (%), buffer concentration (M) and urea concentration (%) for optimization of phytase production

Run	Variable				Phytase activity (U/ml)
	Temperature (°C)	Initial moisture (%)	Citrate- buffer (M)	Urea (%)	
1	-1	-1	-1	1	25.75
2	-1	-1	1	-1	22.32
3	-1	-1	-1	-1	34.69
4	-1	-1	1	1	35.27
5	-1	1	-1	-1	40.04
6	-1	1	1	1	34.51
7	-1	1	-1	1	45.28
8	-1	1	1	-1	24.76
9	1	-1	-1	-1	41.22
10	1	-1	1	1	36.30
11	1	-1	-1	1	65.01
12	1	-1	1	-1	17.64
13	1	1	-1	1	68.55
14	1	1	1	-1	20.06
15	1	1	-1	-1	41.98
16	1	1	1	1	20.73
17	-2	0	0	0	22.72
18	2	0	0	0	40.35
19	0	-2	0	0	36.10
20	0	2	0	0	42.50
21	0	0	-2	0	71.57
22	0	0	2	0	12.53
23	0	0	0	-2	24.62
24	0	0	0	2	39.63
25	0	0	0	0	79.94
26	0	0	0	0	85.35
27	0	0	0	0	93.08
28	0	0	0	0	81.46
29	0	0	0	0	83.10
30	0	0	0	0	86.77

The regression equations obtained after analysis of variance (ANOVA) provided the levels of phytase produced as a function of the values of temperature (A), initial moisture (B), buffer concentration (D) and urea concentration (F). The production of phytase (Z) could be predicted by the model:

$$Z = 84.28 + 7.01A - 26.49A^2 - 22.61B^2 - 22.417D - 21.24 D^2 + 9.89F - 26.20F^2 - 11.64AD$$

Where Z is phytase production (U/ml), A is temperature of fermentation (°C), B is initial moisture (%), D is buffer concentration (M) and F is urea concentration (%). The coefficient of determination is used typically as a good-of-fit-measure and should be close to 1.0 for a good statistical model. For phytase production $R^2=0.937$ ($R_{adj.}=0.9134$) means that 93.7% of experimental data of the phytase production was compatible with the data predicted by the model. Values of Prob>F less than 0.05 indicate model terms are significant while values greater than 0.1000 indicate the model terms are not significant (**Table 9**).

Phytase activity was affected by temperature ($p=0.0246$), initial moisture ($p<0.0001$), Na-citrate buffer ($p<0.011$) and urea ($p=0.02$). An increment in the variables temperature of fermentation (+7.01A) and urea concentration (+9.89F) an increase of enzyme activity, while pH and buffer concentration increment have negative effects on enzyme activity. The effects of each variable are presented in **Table 10**. Among all interactions, only AD interactions showed significance at 5% level as linear and quadratic terms of A, D, F and the quadratic term of B.

In the presence of simple sugars, strong repression of enzyme synthesis was observed. In mould such as *Aspergillus niger*, formation of mycelial pellets in the presence of glucose or fructose as the sole carbon source was shown to be responsible for the low enzyme yields (Shieh and Ware 1968; Han and Gallagher 1987). The phenomenon of glucose repression has been reported in the yeast *A. adenivorans*, which was overcome by replacing glucose with galactose (Sano et al 1999). The regulation of the formation of phytate-degrading enzymes was not suggested to be due to the carbon source itself, but a change in the level of cellular cyclic adenosine monophosphate (cAMP). It has been established that a complex of cAMP with a protein called cAMP receptor protein (CRP) plays a central role in activating and repressing the

expression of many genes (Saier et al 1996). cAMP-CRP is directly involved in the regulation of the formation of phytate-degrading enzymes was shown in some microorganisms (Greiner 2007). Nitrogen source and the content also significantly affected phytase production (**Fig.2**). When no nitrogen source was added in the growth medium, growth and phytase production were greatly affected. Organic nitrogen sources in general supported cell mass production more than inorganic nitrogen sources (Vats and Banerjee 2002). Li et al (2008) found a high yield of extracellular phytase from marine yeast strain in a simple oat-based medium which contained ammonium sulphate 2.3%. According to Kaur and Satyanarayana (2005), phytase titres increased with the increasing in the concentration of urea to produce a cell-bound phytase by *Pichia anomala*.

There is a lack of published data in literature on the effects of buffer and its concentration on phytase production. Na-citrate buffer contain citric acid and Na-sodium acetate and the concentration of 0.3M showed suitable to increase phytase synthesis when compared to experiments on fermentation without using Na-citrate buffer (data not shown). Acetate, phosphate and citrate buffer were tested previously in solid state fermentation composition compared to fermentation with deionized water only. It seems that buffered medium culture ensure pH stability from organic acids production and other metabolites deriving from fermentation. The increase of Na-citrate buffer concentration up the 0.3M affects negatively the phytase production (-22.41 and -21.24) for linear and quadratic term respectively (**Fig. 3**).

Table 9 - Variance Analysis (ANOVA) of significant factors

Variation source	SS	Df	MS	F	p
A (L)	294.91	1	284.911	5.86430	0.0246
A (Q)	4814.60	1	4814.596	95.73824	<0.0001
B (Q)	3507.07	1	3507.070	69.73808	<0.0001
D (L)	3015.27	1	3015.266	59.95856	<0.0001
D (Q)	3093.58	1	3093.576	61.51575	<0.0001
F (L)	587.17	1	587.169	11.67586	0.0259
F (Q)	4707.99	1	4707.990	93.61839	<0.0001
A(L) by (D) L	542.07	1	542.075	10.77916	0.0035
Error	1056.07	21	50.289		
Total	16847.51	29			

Table 10 - Estimation by point, by interval (95%) and hypothesis tests to the effects

	Effect	Std. Err.	T(21)	P	-95% Cnf.Limt	95% Cnf.Limt
Mean/Interc	84.28	2.89	29.11	<0.0001	78.26	90.30
A (L)	7.01	2.89	2.42	0.0246	.99	13.03
A (Q)	-26.49	2.71	-9.78	<0.0001	-32.13	-20.86
B (Q)	-22.61	2.71	-8.35	<0.0001	-28.25	-16.98
D (L)	-22.41	2.89	-7.74	<0.0001	-28.44	-16.39
D (Q)	-21.24	2.71	-7.84	<0.0001	-26.87	-15.60
F (L)	9.89	2.89	3.42	0.00259	3.87	15.91
F(Q)	-26.20	2.71	-9.67	<0.0001	-31.83	-20.57
A(L) x D(L)	-11.64	3.54	-3.28	0.00355	-19.01	-4.26

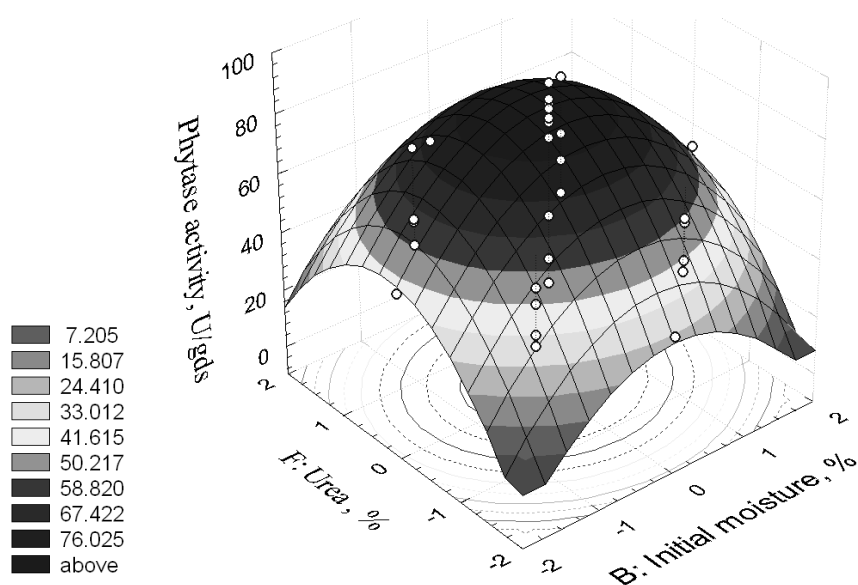


Fig. 2 – Response surface curve of phytase production showing the optimum regions and values for B and F

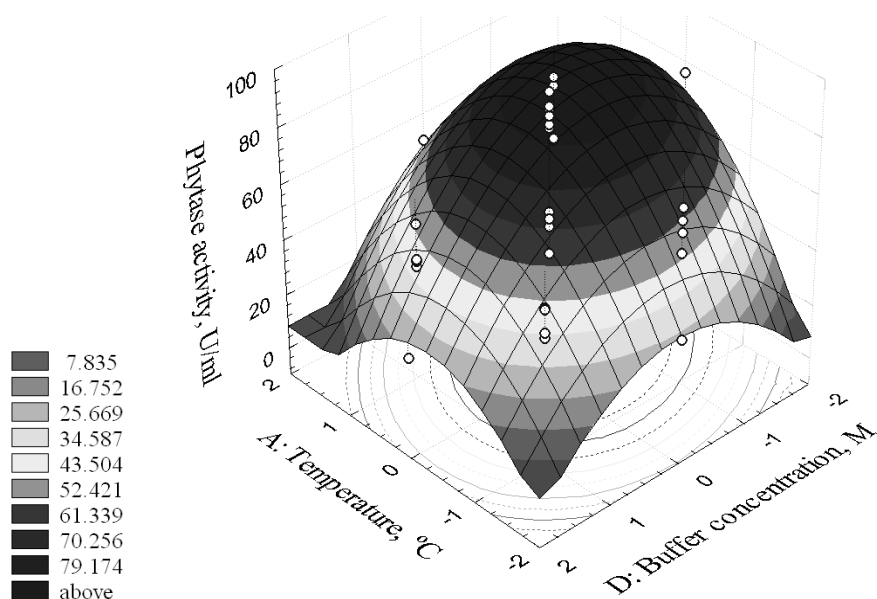


Fig. 3 - Response surface curve of phytase production showing the optimum regions and values for A and D in phytase production

The use of a screening design as Plackett-Burman before building a factorial plan showed to be an important tool to define variables that influence significantly in enzyme production. Li et al (2008) optimized phytase production by *Kodamaea ohmeri* in oats medium achieving an overall 9-fold enhancement in phytase activity; Singh & Satyanarayana (2008) improved phytase production in SSF by *Sporotrichum thermophile* with an overall 2.6-fold improvement due to optimization using sesame oil cake as the substrate; Kaur & Satyanarayana (2005) achieved higher phytase production in molasses medium by *Pichia anomala* attained an overall 7.5-fold enhancement due to optimization. By using statistical optimization, 1.3-fold increasing in phytase production by *Rhizomucor pusillus* (Chadha et al., 2004), 1.85-fold higher phytase production in *M. racemosus* (Bogar et al., 2003a) and 1.67-fold higher phytase production by *A. ficum* NRRL3135 (Bogar et al., 2003b) were achieved in SSF.

A plot of phytase production in the course of fermentation time before and after optimization is presented below (**Fig. 4**). An overall 4.3-fold improvement in phytase production was achieved, considering the production which peaks at 96 hours of fermentation.

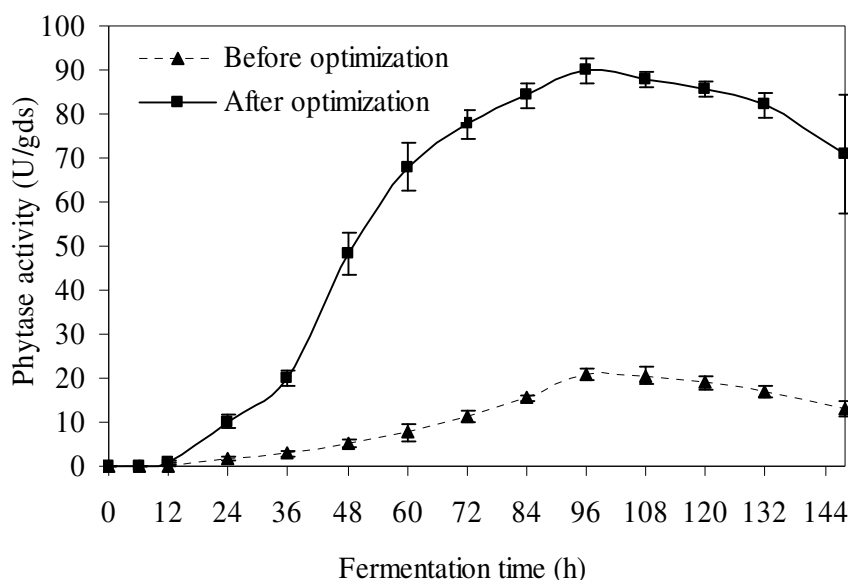
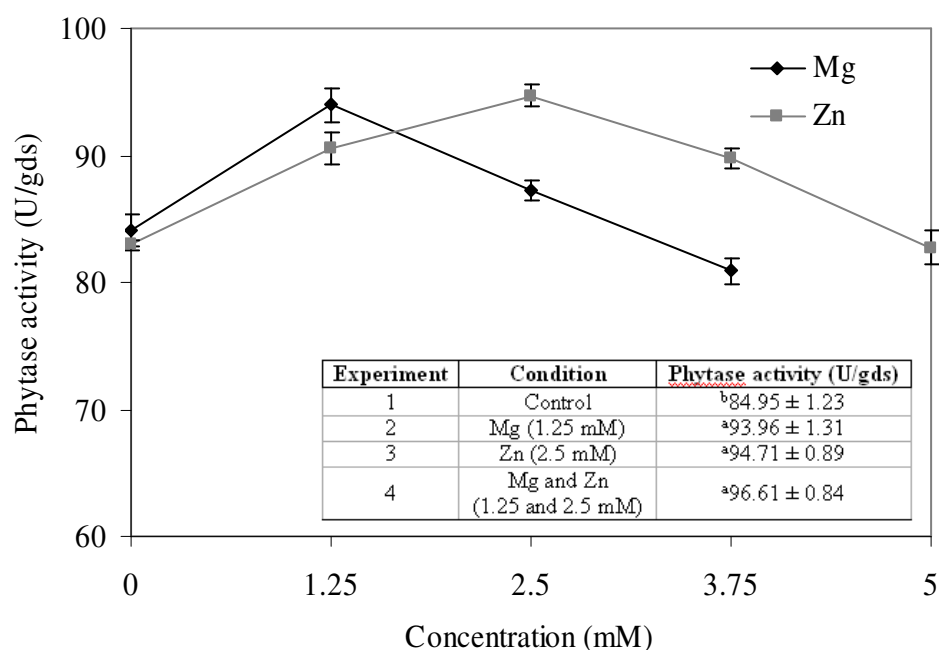


Fig. 4 – Phytase production in the course of fermentation before and after optimization

Brans are considered excellent substrates for the production of extracellular phytate-degrading enzymes in microorganisms. As phytate in bran is less soluble than sodium phytate, phosphate concentrations are lower due to a slower release from bran phytate, and therefore repression of enzyme synthesis by phosphate is reduced. This ensures a continuous production of phytate-degrading enzymes during the whole fermentation process (Greiner 2007).

Effect of salts in phytase production

Mg, Zn, K and Fe salts showed significant effect in phytase production according to Plackett-Burman design ($R^2=0.98$ and $R_{adj}=0.91$). **Fig. 5** shows the influence of Mg and Zn on phytase synthesis during citric pulp fermentation by *A. niger* FS3.



Treatments with the same letters at the column do not differ significantly according to the totally randomized test

Fig. 5 – Influence of Mg and Zn on phytase synthesis

After addition of Mg 1.25 mM and Zn 2.5 mM, phytase yield increased 12% compared to optimized conditions (control). A totally randomized design indicated that treatments (experiment 2, 3 and 4) differs from control (experiment 1) significantly ($p < 0.05$). In one-variable-at-a-time approach studies, K, Fe, Mg and Zn showed significant difference, but Plackett-Burman tool indicated that only magnesium and zinc are significant elements. It may be explained that due to the presence of these elements naturally in citric pulp or the repression of phytase synthesis overdue the presence of the others elements. It is in accordance with studies of In et al (2008) which phytase produced by *S. cerevisiae* CY was improved by the presence of $MgSO_4$. Singh & Satyanarayana (2008) also found magnesium sulphate as important factor in phytase production by a thermophilic mould *Sporotrichum thermophile* in cane molasses medium.

4. Conclusions

Citric pulp bran as substrate in inoculum medium and in solid state fermentation may show a cost-effective process to phytase production instead the use of phytate as component media. Optimization of phytase production by the fungus *A. niger* FS3 in citric pulp fermentation resulted in overall 4.3-fold increase of phytase production using statistical tools which the best parameters optimized were 30°C, initial pH 5.0, 0.3M Na-citrate buffer concentration, 65% initial moisture and 1.5% urea. Besides, magnesium and zinc showed importance in phytase production, contributing to an increase of 12% in enzyme yield after optimization studies.

References

IBGE, Brazilian Institute of Geography and Statistic. [On line] <<http://www.ibge.gov.br>> (2007).

Abecitrus, The Brazilian Association of Citrus Exporters. [On line] <<http://www.abecitrus.com.br>> (2008).

Nelson PE, Tressler DK (1980), Fruit and Vegetable Juice Processing Technol., AVI Publishing Co., Westport, USA, 603p.

Spier MR, Greiner R, Rodríguez-León JA, Woiciechowski AL, Pandey A, Soccol VT, Soccol CR (2008), Phytase production using citric pulp and other residues of the agro-industry in SSF by fungal isolates, Food Technol. Biotechnol. 46 (2008), 178-182.

Citroex, Concentrates and Essential Oils Company, Brazil (2008). [On line] <<http://www.citroex.com.br>> (2008).

Oliveira NJF, Melo MM, Lago LA (2004), Pesticides, aflatoxins and macro and microminerals analyses of commercial citrus pulp pellets. Arq. Bras. Med. Vet. Zootec., 56 (5), 679-682.

Vats P, Banerjee UC (2002), Studies on the production of phytase by a newly isolated strain of *Aspergillus niger* var teigham obtained from rotten wood-logs, *Process Biochem.* 38, 211-217.

Quan CS, Zhang LH, Wang YJ, Ohta Y (2001), Production of phytase in a low phosphate medium by a novel yeast *Candida krusei*, *J. Biosci. Bioeng.* 92, 154–60.

Vats P, Banerjee UC (2006), Catalytic characterization of phytase (*myo* - inositolhexakisphosphate phosphohydrolases) from *A. niger* Van Teighem: Glycosylation pattern, kinetics and molecular properties, *Enzyme Microb. Technol.* 39, 596–600.

Andlid T, Veide J, Sandberg AS (2004), Metabolism of extracellular inositol hexaphosphate (phytate) by *Saccharomyces cerevisiae*. *International Journal of Food Microbiology* 97, 157– 169.

Soni SK, Khire JM (2007), Production and partial characterization of two types of phytase from *Aspergillus niger* NCIM 563 under submerged fermentation conditions. *World J. Microbiol. Biotechnol.* 23: 1585-1593.

Kim YO, Kim HK, Bae KS, Yu JH, Oh TK (1998), Purification and properties of a thermostable phytase from *Bacillus* sp. DS11. *Enzyme Microbial. Technol.* 22: 2-7.

Bhavsar K, Shah P, Soni SK, Khire JM (2008), Influence of pre-treatment of agriculture residues on phytase production by *Aspergillus niger* NCIM 563 under submerged fermentation conditions. *Afr. J. Biotechnol.* 7: 1101-1106.

Quan CS, Tian WJ, Dan SD, Kikuchi JI (2004), Purification and Properties of a Low-Molecular-Weight Phytase from *Cladosporium* sp. FP-1, *J. Biosci. Bioeng.* 97, 260–266.

Li XY, Liu ZQ, Chi ZM (2008), Production of phytase by a marine yeast *Kodamaea ohmeri* BG3 in an oats medium: Optimization by response surface methodology, *Bioresource Technol.* 99, 6386–6390.

Singh B, Satyanarayana T (2006), A marked enhancement in phytase production by a thermophilic mould *Sporotrichum thermophile* using statistical designs in a cost-effective cane molasses medium. J. Appl. Microbiol. 101 (2), 344–352.

Chadha BS, Harmeet G, Mandeep M, Saini HS, Singh N (2004), Phytase production by the thermophilic fungus *Rhizomucor pusillus*. World J. Microbiol. Biotechnol. 20, (1), 2004.

Plackett RL, Burman JP. (1946), The design of optimum multifactorial experiments. Biometrika, 33, 305–325.

Lotfy WA, Ghanem KM, El-Helow ER (2007), Citric acid production by a novel *Aspergillus niger* isolate: II. Optimization of process parameters through statistical experimental designs. Bioresource Technol. 98, 3470–3477.

Heinonen JK, Lahti RJ (1981), A new and convenient colorimetric determination of inorganic orthophosphate and its application to the assay of inorganic pyrophosphatase, Analytical Biochem. 113, 313-317.

El-Batal AI, Karem HA (2001), Phytase production and phytic acid reduction in rapeseed meal by *Aspergillus niger* during solid state fermentation. Food Research International 34, 715–720.

Roopesh K, Ramachandran S, Nampoothiri KM, Szakacs G, Pandey A (2006), Comparison of phytase production on wheat bran and oilcakes in solid-state fermentation by *Mucor racemosus*, Bioresource Technol. 97, 506–511.

Chantasartasamee K, Ayuthaya DI, Intarareugsorn S, Dharmsthiti S (2005), Phytase activity from *Aspergillus oryzae* AK9 cultivated on solid state soybean meal medium. Process Biochemistry, 40, 7, 2285-2289.

Ramachandran S, Roopesh K, K. M. Nampoothiri, G. Szakacs, A. Pandey (2005), Mixed substrate fermentation for the production of phytase by *Rhizopus* spp. using oilcakes as substrates. Process Biochemistry 40 (5), 1749-1754.

Greiner, R. (2007), Inositol Phosphates: Linking Agriculture and the Environment. Phytate-degrading enzymes: regulation of synthesis in microorganisms and plants (3) 78-95.

Singh B., Satyanarayana T (2008), Phytase production by a thermophilic mould *Sporotrichum thermophile* in solid state fermentation and its potential applications. Bioresource Technol. 99, 2824–2830.

Parvinder K, Satyanarayana T (2005), Production of cell-bound phytase by *Pichia anomala* in an economical cane molasses medium: Optimization using statistical tools. Process Biochemistry 40, 3095–3102.

Vohra and Satyanarayana (2003), Phytases: Microbial sources, production, purification, and potential biotechnological applications. Critical Reviews in Biotechnol. 23(1), 29-60.

Sano K; Fukuhara H.; Nakamura Y. (1999), Phytase of the yeast *Arxula adeninivorans* Biotechnology Letters 21, 33–38.

Chadha, B.S., Gulati, H., Minhas, M., Saini, H.S., Singh, N (2004). Phytase production by the thermophilic fungus *Rhizomucor pusillus*. World J. Microbiol. Biotechnol. 20, 105–109.

Bogar B, Szakacs G, Linden JC, Pandey A, Tengerdy RP (2003a). Optimization of phytase production by solid substrate fermentation. J. Ind. Microbiol. Biotechnol. 30, 183–189.

Bogar B, Szakacs G, Pandey A., Abdulhameed S., Linden JC, Tengerdy RP (2003b). Production of phytase by *Mucor racemosus* in solid-state fermentation. Biotechnol. Progr. 19 (2), 312–319.

Han YW, Gallagher DJ, Wilfred AG (1987), Phytase production by *Aspergillus ficuum* on semi solid substrate. J. Ind. Microbiol. 2, 195-200.

Shieh TR, Ware JH (1968), Survey of microorganisms for the production of extracellular phytase, Appl Environ Microbiol. 1968 September; 16(9), 1348-1351.

In MJ, Seo SW, Oh NS (2008), Fermentative production and application of acid phytase by *Saccharomyces cerevisiae* CY strain African Journal of Biotechnology 7 (17), 3115-3120.

Singh B, Satyanarayana T.(2006), A marked enhancement in phytase production by a thermophilic mould *Sporotrichum thermophile* using statistical designs in a cost-effective cane molasses medium J. Applied Microbiol. 2006, 101 (2), 344-352

Saier MH Jr, Ramseier TM, Reizer J (1996) Regulation of carbon utilization. In: Neidhardt FC, Curtiss R III, Ingraham JL, Lin ECC, Low KB, Magasanik B, Reznikoff WS, Riley M, Schaechter M, Umberger HE (eds) *Escherichia coli* and *Salmonella*: Cellular and Molecular Biology, 2nd edn ASMP Press, Washington DC, 1325-1343.

CHAPTER III

MONITORATION OF RESPIROMETRIC ACTIVITY AND ITS RELATION WITH PHYTASE PRODUCTION, FUNGAL GROWTH AND FORCED AIR IN COLUMN-TYPE BIOREACTOR

Abstract

Respirometric data of phytase recovered from citric pulp fermentation in column-type bioreactor was monitored using a new data acquisition system. There are a considerable number of studies concerning phytase production in laboratory-scale, but a lack of data of its production in other types of bioreactor. Respirometric activity during fermentation and its relation with fungal growth, forced air and enzyme production using a column-type bioreactor were objectives of this work. Phytase synthesis by a new fungus isolated from soil, *A. niger* FS3, increased with forced air. The O₂ consumption and CO₂ production during solid-state fermentation were monitored by sensors (in the bottom and top of the columns) linked to controllers, recorded by an acquisition software and processed by Fersol2[®] software tool. Phytase synthesis was associated to fungal growth, and therefore could be used to estimate biomass formed in citric pulp fermentation.

Keywords: phytase, respirometry, citric pulp, solid-state fermentation

1. Introduction

Nowadays enzyme production is a growing field of biotechnology with annual world sales close to two billion dollars, in which the market volume of phytases is in the range of €150 million and increasing (Greiner and Konietzny, 2006). Citric pulp is one of the major agro industrial residues in Brazil. It is obtained from citrus processing industry and today it has application not only in feed: as a substrate for fermentation, it may be used for production of organic acids production, aroma compounds, and enzymes (Soccol et al 2002).

Phytases (*myo*-inositol hexakisphosphate phosphohydrolases; EC 3.1.3.8 and EC 3.1.3.26) are enzymes capable of initiating the stepwise hydrolysis of phytate (*myo*-inositol (1,2,3,4,5,6) hexakisphosphate) (Greiner 2007; Pandey et al 2001; Mitchell et al 1997). Phytate hydrolysis is desirable in order to improve mineral bioavailability (Sandberg 1988; Greiner 2007), because phytate has a strong affinity to chelate multivalent metal, especially iron, zinc, calcium, and magnesium. The chelates thus formed, give rise to insoluble salts with poor absorption characteristics and hence low bioavailability (Hurrell 2004). Low absorption of these nutrients from cereals is considered as a factor in the aetiology of mineral deficiencies.

On the other hand, phytases release phosphorus from organic sources, turning phosphate available as a nutrient. This characteristic is important, since phosphorus is a key component in agricultural fertilizers and its lack would affect future soil quality and production (Mitchell 2007).

There has been a great interest to find application of organic residues from agriculture and industries over the past years (Ramachandran et al 2007). Residues such as bran, husk, bagasse, citric pulp and seeds are utilized as a potential raw material in bioprocesses, since they provide excellent conditions for the growth of microorganism, supplying the essential nutrients for them (Pandey and Soccol 1998, 2000; Pandey 1994; Pandey et al. 2000a,b, 1999, Pandey 1992), besides reducing costs of industrial production and adding value to agro-industrial residues (Ramachandran et al 2007), minimizing the pollution caused by these residues (Macedo and Fraga 2007) but also helps solving pollution problems and economically useful to produce enzymes, organic acids, pigments, flavor and aroma compounds, mushrooms, pigments, polysaccharides, hormones, human food and animal feed (Soccol and Vandenberghe 2003). Solid state fermentation (SSF) is the most adequate fermentation process to benefit from the use of

these residues directly or with some previous pre-treatment. SSF is defined as any fermentation process performed on a non-soluble material that acts both as physical support and source of nutrients in absence of free flowing liquid (Pandey et al 1999, Soccol & Vandenberghe 2003; Macedo & Fraga 2007; Moo-Young et al 1983). Although SSF has gained importance due to several advantages over submerged cultivations (Singhania et al 2008, Doelle and Mitchell 1992), there are some disadvantages that hinder the optimal application and scale up of this process. Monitoring and controlling cultivation parameters such as biomass content, pH, temperature and moisture can lead to difficulties because of heterogeneous composition of substrate, lack of free water and low conductivity of solid particles (Singhania et al 2008, Sturm et al 2007, Mitchell et al 1991). In SSF, the kinetic process behavior is very difficult to analyze. It is due to the impossible separation of biomass from the solid substrate and because of its heterogeneous characteristic (Singhania et al 2008, Pandey et al 2001). The interest in studying kinetics lies on the fact that it allows many important parameters determination such as specific growth rate, process yield and productivity, heat evolved, process control criteria, strategy for the production of a particular product, and scale-up considerations (Pandey et al 2001).

The biomass synthesis is one of the most important processes present in a fermentation bioprocess. In submerged fermentation process, this parameter can be normally measured through direct methods such as cell counting, dry biomass or optical density determination. Nevertheless, in SSF the proper characteristic is that the biomass will usually be attached to the solid surface, hence, it is not so easy to measure it directly. Some indirect measurements of biomass in SSF have been used, because many SSF processes involve filamentous fungi and its bind tightly to the substrate. The detection of some cell compounds such as protein other cell component such as DNA, RNA, chitin, ergosterol and glucosamine (Raimbault 1981, Okasaki et al. 1980, Matchman et al 1985, Desgranges et al 1991, Matcham et al 1985, Carvalho et al 2006), or the measurement of metabolic data such as CO₂ evolution rate and O₂ consumption rate (Bellon-Maurel 2003, Spier et al 2007, Nishio et al 1979, Carrizalez et al 1981) has distracted the attention from growth kinetics and the mathematical modeling of heat and mass transfer in SSF (Doelle et al 1992). Some kinetic models are used to describe the growth kinetics in SSF (Rodriguez-Leon et al 1988, Viccini et al 2001, Hamidi-Esfahani et al 2004, Carvalho et al 2006, Spier et al 2007).

There are limitations of data about phytase microbial in literature concerning the type of bioreactor employed, influence of aeration in phytase synthesis, respirometric activity during fermentation. All references cited in **Table 1** show results of phytase production performed in flasks of laboratory-scale, which demonstrate a lack of studies about fermentation respirometry.

Table 1 - Fungi phytase produced by SSF as reported in the literature

Microorganism	Substrate	Phytase activity Equivalent* (U/gds)	References
<i>A. niger</i>	Dry olive waste and rock phosphate	58	Vassilev et al (2007)
<i>Sporotrichum thermophile</i>	Sesame oil cake	20.8	Singh and Satyanarayana (2007)
<i>Mucor racemosus</i> NRRL 1994	Wheat bran and sesame oil cake	44.5	Roopesh et al (2006)
<i>A. oryzae</i> AK9	Soybean meal	30	Chantasartrasamee (2005)
<i>R. oryzae</i> NRRL 1891	Coconut oil cake and sesame oil cake	64.0	Ramachandran et al (2005)
<i>A. oryzae</i>	Steamed brown rice	10	Fujita et al (2003)
<i>A. ficcum</i> TUB F-1165	Polystyrene beads and glucose	10.07	Gautam et al (2002)
<i>R. oligosporus</i>	Coconut oil cake	14.29	Sabu et al (2002)
<i>A. niger</i> A-98 isolate	Rapeseed meal	130	El-Batal and Kareem (2001)
<i>A. niger</i> (Alltech Inc.)	Wheat bran and soy bean meal	167	Papagianni et al (1999)
<i>A. ficcum</i> NRRL 3135	Canola meal	100	Ebune, Al-Asheh, Duvnjak (1995)
<i>A. ficcum</i> NRRL 3135	Wheat bran	25	Bogar et al (2003)

*Activities reported in literature were adjusted to 1U per gram of dry substrate. One unit is defined as the amount of enzyme releasing 1 μmol of inorganic phosphorus per ml per min under the assay conditions.

This work presents a new process control strategy to measure environmental variables such as process temperature, flow rate, humidity, O_2 uptake rate and CO_2 production rate in the inlet and outlet gas with the aim of studying the respirometric activity and its relation with phytase production, fungal growth and forced air in a column-type bioreactor.

2. Materials and Methods

2.1 Microorganism and inoculum preparation

A. niger FS3 was isolated from soil samples (Spier et al. 2008). After growth in PDA slants at 30°C for 8 days, the strain was stored at 4°C for further use. The strain was reactivated in sterile CP broth, incubated in shaker at 30°C / 72h / 120rpm. CP broth contained citric pulp <0.8mm particles size, diluted 10-fold in distilled water adjusted to pH 5.0. After filtered with Whatman n°1 paper and autoclaved, the broth was diluted to achieve sugar concentration of 2.5% (w/v) and 0.3% w/v ammonium nitrate was added.

2.2 Effect of aeration and moisture in columns

Solid-state fermentation was performed in a glass column bioreactor (20 cm x 4 cm) using 0.8-2.0mm particles of citric pulp (Cargill, Brazil). The solution composed by 0.33M Na-citrate buffer pH=5.0 contained: urea 1.5%, adjusted to 60 to 70% of initial moisture and aeration rate (0.8, 1.0 and 1.2 NmL/g.min) according to 3² factorial design (Table 2). A control (C) was incubated without forced air. 10% v/v of *A. niger* FS3 pellets suspension growth in 10% (w/v) citric pulp solution media was inoculated and thoroughly mixed and added to the columns. Each column contained 68-70 g of citric pulp medium. The columns were capped at both ends with cotton filters. They were connected to humidifiers and immersed in a water bath at 30°C/96h. This experiment involved 2 factors; initial moisture (%) and aeration rate (NmL/g.min) at three different levels using a 2³ factorial design (**Table 2**). Extracellular crude phytase entailed initial maceration, filtration through Whatman n°1 filter paper and the filtrate was centrifuged (4500 rpm/15min /4°C). The supernatant was used to phytase assay.

Table 2 - Codified and uncoded levels of the independent variables used in experimental design to estimate higher phytase synthesis in column-type bioreactor

Factors	Range and levels		
	-1	0	+1
Initial moisture (%)	60	65	70
Aeration rate (NmL/g.min)	0.6	1.0	1.4

2.3 Respirometric measurements

Column-type bioreactor fermentation was used to determine respiration activity during *A. niger* FS3 growth and phytase synthesis. Ten columns with 85 g of media were prepared as described previously. Fermentation parameters were monitored and controlled with a PC using a new data acquisition system in real time developed under LAquis platform (LCDS, Brazil). O₂ and CO₂ parameters monitored at the inlet and outlet (off-gas) of the column were measured with a oxygen sensor (sensor O₂-A₂) (Alphasense Ltd. UK) and carbonic oxide sensor (sensor CO₂-A1) (Alphasense Ltd. UK,) oxygen consumption, dioxide carbon production, phytase activity, biomass, process temperature, air flow and respirometric quotient during solid state fermentation, similar to (Spier et al 2007, Carvalho et al 2006), but using sensors to get data and controllers to adjust set point of variables process temperature (°C) and aeration rate (NmL/g.min), (Fersol 2 system) (Sturm et al. 2007) instead of using gas chromatography system (Fersol 1) (Rodríguez-León 1988). To transfer data from O₂ and CO₂ sensors, process temperature, relative humidity, and flow rate, PID controllers were used and connected in a Modbus protocol net. Each parameter was read by sensor, transferred to the controller and connected to the acquisition software.

Each 24 hours columns were taken off to analyze biomass and phytase activity. Cell biomass was experimentally determined by ergosterol quantification (Seitz et al 1979; Carvalho et al, 2006). Kinetic parameters as oxygen uptake rate, volumetric flow of CO₂ (L/h) were calculated by balance analysis as described by (Prado et al. 2005, Rodriguez-Leon et al. 1988).

Process universal controller (Novus) indicated and controlled parameters of solid state fermentation. It interfaces the parameters to ModBus network and to the computer. A transmitter RHT-DM 4-20mA 150mm (Novus) measured air humidity and the outlet process temperature (**Fig.1**).

2.4 Biomass determination in SSF

Samples of 1g of fermented substrate, from each column were used. The biomass was estimated by ergosterol extraction based on Seitz method (Seitz et al, 1979) with some modifications. The method consisted in a mixture composed by 1 g of sample, 4 mL of ethanol and 2 mL of NaOH 2M incubated in a water bath at 70°C per 1h. 4 mL of HCl 1M were added and agitated. 2 mL of KHCO₃ 1M and 4 mL of n-hexane were

agitated vigorously and then centrifuged at 4500 rpm per 5 min. The supernatant were collected in a light-protected and open flask for solvent evaporation. A second centrifugation occurred after the addition of 4 mL of n-hexane at 4500 rpm per 3 minutes. A last centrifugation (4500 rpm/2 min) was performed after the addition of 2 mL of n-hexane. The extracts were analyzed in an HPLC Varian ProStar with a C₁₈ column and a photodiode array detector set to 282 nm. An injection volume of 10 µL of the sample was used. The mobile phase used was pure methanol (from 0 to 3 min), pure acetonitrile from 3 to 10 minutes, and pure methanol from 10 to 15 minutes with a flow of 1 mL/min, for the elution of other eventual components of sample. As standard, it was used an ergosterol PA solution (10000µg/mL), with dilutions to 5000 and 1000 µg/mL. The baseline was determined with 10 µL of pure chloroform.

2.5 Biomass prediction

Biomass prediction at a certain time (X_n) was estimated from O₂ metabolism. The estimation was performed by assuming values for biomass yield based on oxygen consumption ($Y_{X/O}$) and biomass maintenance coefficient (m_x). Biomass was estimated with the Fersol2 software which uses updated techniques to manipulate all the parameters (Sturm et al 2007). Fersol2 uses a mathematical set of methods called Numerical Calculus, able to calculate the integral and differential equations. To evaluate the total oxygen consumed, it is useful the integral equation described by (Pandey et al 2001), as the equation showed below.

$$\int_{t=0}^{t=n} \frac{dO_2}{dt} dt$$

After adjusting the biomass values, it estimates process indicators as: process yield based on consumed oxygen for biomass synthesis, specific growth rate at the logarithmic or exponential phase, and maintenance coefficient (Sturm et al 2007).

From the Numerical Calculus, the numerical integration is performed by the Trapeze Rule, which leads to a discrimination of the theoretical equation, but in this specific case the values are taken from a table of oxygen quantity consumed, hence it is not quite a problem. The result is equivalent to the area under the oxygen consumption curve, determined by some periodic measures. On the other hand, some problems need

an iterative method to solve. To determine the biomass (X_n), making use of Metabolic Gas Balance Method described in (Pandey et al. 2001), it is necessary to make an adjust in the biomass curve using the following equation:

$$X_n = \frac{\left\langle Y_{X/O} \Delta t \left\{ \frac{1}{2} \left[\left(\frac{dO_2}{dt} \right)_{t=0} + \left(\frac{dO_2}{dt} \right)_{t=n} \right] + \sum_{i=1}^{i=n-1} \left(\frac{dO_2}{dt} \right)_{t=i} \right\} + \left(1 - \frac{a}{2} \right) X_0 - a \sum_{i=1}^{i=n-1} X_i \right\rangle}{\left(1 + \frac{a}{2} \right)}$$

Where $a = m \cdot Y_{X/O} \cdot \Delta t$. Taking into account some real measures of the biomass at a determined time and the initial process conditions, a kind of trial and error method is used to adjust the intermediary values. Numerical Calculus presents many iterative methods to solve this kind of problem because the trials should not be chosen randomly, it is necessary to maximize the convergence and it allows to use some stop criteria to the number of iterations. Software development tools allow this kind of calculation without additional care (Sturm et al 2007).

2.6 Phytase activity assay

Phytase activity was determined by measuring the amount of liberated inorganic phosphate based in a modified method of (Heinonen and Lahti 1981). 350 μ L substrate (2.5mM sodium phytate (Sigma) in 0.2 M sodium acetate buffer, pH 4.5) and 50 μ L enzyme were co-incubated at 50°C for 30 minutes. The reaction was stopped by addition of 1.5 ml of AAM-solution, using 10 mM ammonium molibdate:5N H_2SO_4 :acetone in the proportion 1:1:2 and 100 μ L of citric acid. One unit of enzyme activity was defined as the amount of enzyme capable to release one μ mol of inorganic phosphate per min under defined reaction conditions.

All analysis was performed in triplicate and the mean values and standard deviation are reported.

3. Results and Discussion

The assay in column-type bioreactor consisted in two fermentations using citric pulp. The first experiment consisted in evaluating the adequate aeration flow and initial moisture for phytase synthesis in columns; then a respirometric data analysis was performed (Fig.1).

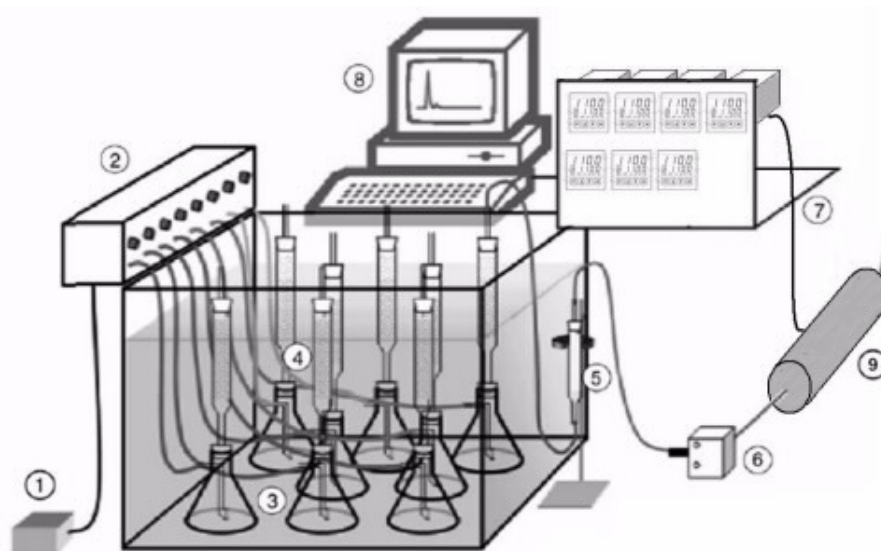


Fig.1 - 1) air pump; 2) air distribution system; 3) humidifiers; 4) fermentation columns immersed in a water bath with controlled temperature; reactor; 5) filter; 6) flow sensor; 7) controllers display; 8) computer with data acquisition and control software; 9) cylindrical sensors base, where are installed the sensors: CO₂ and O₂, humidity and outlet temperature.

3.1 Effect of initial moisture and aeration rates in column

SSF was carried out in glass column-type bioreactor at 30°C with different aeration rates and initial moisture. Fermented medium was analyzed for phytase activity after 96 hours of fermentation. The results of 3² factorial design (**Table 3**) indicates higher level of phytase production at 65% of initial moisture and air flow at 1.0 Nml/g.min i.e, 1.0 mL of air in standard conditions per gram of wet substrate per minute. The factors initial moisture (Q) and air flow (L) demonstrated significant effect on phytase synthesis.

Table 3 - Results of 3² factorial design for two variables: initial moisture (%) and aeration rate (NmL/g.min) for optimization of phytase production in column-type bioreactor

Run	Initial moisture (%)	Aeration rate (NmL/g.min)	Phytase activity (U/gds)
1	0	1	90.11
2	1	0	82.15
3	-1	-1	66.34
4	-1	0	75.34
5	1	1	82.85
6	0	-1	76.58
7	1	-1	80.75
8	-1	1	79.64
9	0	0	93.73
10	0	0	92.42
11	0	0	96.57
Control	0	-	84.26
$R^2 = 0.87$ and $R_{adj.} = 0.78$			

Air flow at 1.2 NmL/g.min showed similar results comparing to 1.0. Although aeration is also fundamental for aerobic organisms, high aeration rate may interfere negatively in enzyme synthesis, because there is a change in fungal metabolism (Spier et al 2007). Oxygen is an important culture parameter for fungal growth, as the fungal mycelium develops on a solid surface, the void spaces between the hyphae can either be fully or partially filled with water. In the former case, resulting in severe oxygen limitation leading to anaerobic conditions affecting the performance of SSF (Oostra et al 2001, Raghavarao et al 2003). Similar behavior was observed by (Spier et al 2007), and the cause of the reduced phytase production has to be clarified in further studies. Although temperature, initial moisture and forced air were controlled, the phytase synthesis was 10.59% higher with forced air (1.0 NmL/g.min and 65% of initial moisture) achieving the average of 94.24 U/gds than without forced air (84.26 U/gds).

Between initial moisture content studied, the best production of phytase occurred at central point (94.24 U/gds, in average), with an initial moisture of 65% (0) and air flow of 1.0 NmL/g.min (0), but higher aeration (+1) showed similar results of enzyme production (90.11 U/gds). Phytase production decreased significantly at 60% of initial moisture and above 70%.

3.2 Respirometric analysis in column

Information regarding modeling in SSF has been limited due to unavailability of a suitable method for direct measurement of microorganism growth, difficulty to separate it from the substrate, and to determine the substrate utilization rate. Among the several approaches to tackle this problem, an important synthetic substrate model has been used. It is well known that the fermentation kinetics is extremely sensitive to the variation in ambient and internal gas compositions. So, the cellular growth microorganisms can be determined by measuring the change in gaseous compositions inside the bioreactor (Singhania et al 2008).

Parameters such as O₂ consumption, production of CO₂ and the respiratory quotient Q are important to understand fungal growth and its respiration. They were monitored to evaluate the respirometric activity during fermentation, to understand a possible relation between fungal growth and enzyme production. In addition, peaks of respiration during fermentation could be evaluated and it might be possible to define a fermentation stage as a lag, log, or decline growth phase. Furthermore, O₂ consumption rate gives information about oxygen transfer in the solid media (Singhania et al 2008, Spier et al 2007, Carvalho et al 2006, Rodríguez-León 1988). The data obtained of O₂ consumption, CO₂ production, biomass development and activities of phytase are showed in Fig. 2a, 2b and 3.

An adaptation phase was verified within the first 15 hours of fermentation (Fig. 2a and b). There was no significative O₂ consumption and CO₂ production during this period. In addition, biomass (0.10g) as well as the activity of phytase was low (0.9-1 U/gds) and respiratory quotient (Q) was zero. This period may be explained by the adaptation of the fungus to the culture medium.

A shift of respiration was detected after 15 hours of fermentation (Fig. 3a and b), characterizing a logarithm phase from 16 to 48 hours. A plot of ln (X) versus time (h) (**Fig. 2**) is observed a linear region indicating log phase of biomass growing,

considering estimated. A linear equation of log phase is $y=0.0596x-3.172$, where angular coefficient (a) as the inclination indicates the parameter specific growth rate (μ) (h^{-1}) = (tg α = μ) with the same value ($\mu = 0.05h^{-1}$) obtained in Fersol2 presented in **Table 4**. After 48 hours (**Fig.2**), the inclination tends to decrease, which demonstrates a decrease of specific growth rate (μ)

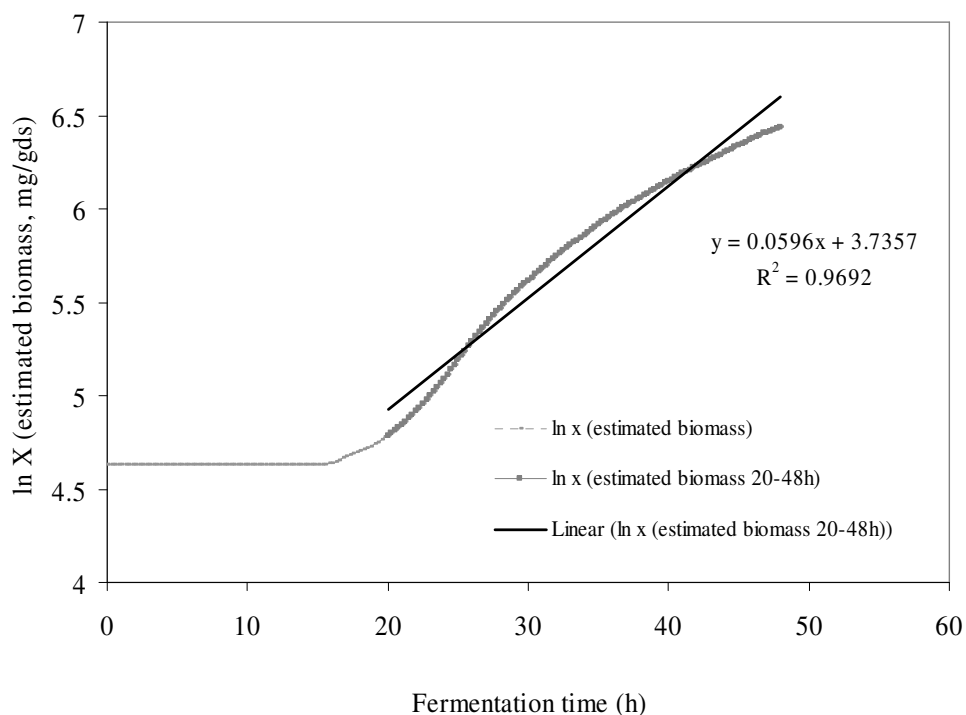


Fig. 2 - Plots of ln (X) showing linearity stage in the log phase of *A. niger* FS3 growth, where X=biomass (mg/gds)

O₂ consumption increased during fermentation achieving a maximum consumption with a concomitant CO₂ production (0.17g/h and 0.29 g/h), respectively at 45.75 hours of fermentation. The biomass content increased during fermentation reaching a maximum value (0.9216 g/gds) at 96 hours of fermentation (Fig. 3). Phytase synthesis also increased during this period with fungal biomass reaching maximal production at

96 hours (94-96 U/gds). A considerable respiratory activity was observed as a result of fungal metabolic activity. Therefore, enzyme synthesis can be assumed as growth associated due to a practically constant respiration quotient Q (**Figure 3a and 3b**) with a value around 1.8. Other studies also showed phytase associated with growth (Krishna and Nokes, 2001). According to Greiner (2005), in moulds phytase production is growth associated, and enzyme activity increases from the onset of growth to the beginning of the stationary phase. However, the peak synthesis of *A. niger* FS3 phytase occurred in the stationary phase, maybe because of the limited condition in nutrient or energy, known to occur in the stationary phase, which could be responsible for phytase induction. A limitation of inorganic phosphate could be also the reason to induce phytase production. Citric pulp presents low inorganic phosphate content (0.025 μ mol/g citric pulp) observed by Spier et al (2008) and Shieh et al (1969) the production of extracellular fungal phytase was induced by a limiting concentration of inorganic phosphate in the growth medium. This characteristic may be assumed in the substrate used in this study, where citric pulp presents low inorganic phosphorus content. The enzyme yield declined during further incubation, which could have been due to the reduced nutrient level of the medium, presence of inorganic-P in high level and the consequent decline phase of *A. niger* FS3.

Phytase increased more than 90 times (from 1.0 to 96 U/gds) during biomass growth. Phytase production reported in the literature shown that 58 U/gds were achieved in dry olive waste (Vassilev et al 2007). Singh and Satyanarayana (2007) obtained 20.8 U/gds by *Sporotrichum thermophile* using sesame oil cake. *Mucor racemosus* NRRL 1994 synthesized 44.5 U/gds in wheat bran and sesame oil cake (Roopesh et al 2006). *A. ficcum* NRRL 3135 produced 25 U/gds using wheat bran as substrate (Bogar et al 2003), and higher phytase yield (130 U/gds) was obtained using rapessed meal by a *A. niger* A-98 local isolate (El-Batal and Karem 2001).

The aeration, however, should be sufficient to guarantee aerobic development of the small amount of biomass present in this log phase. The respiratory quotient Q showed high values in this phase (around 1.8) between 16 and 48 hours of fermentation (log phase). After 48 hours a stationary phase may be observed when respirometric coefficient (QR) decreased slowly, assuming value around 1.0 at 90 hours of fermentation.

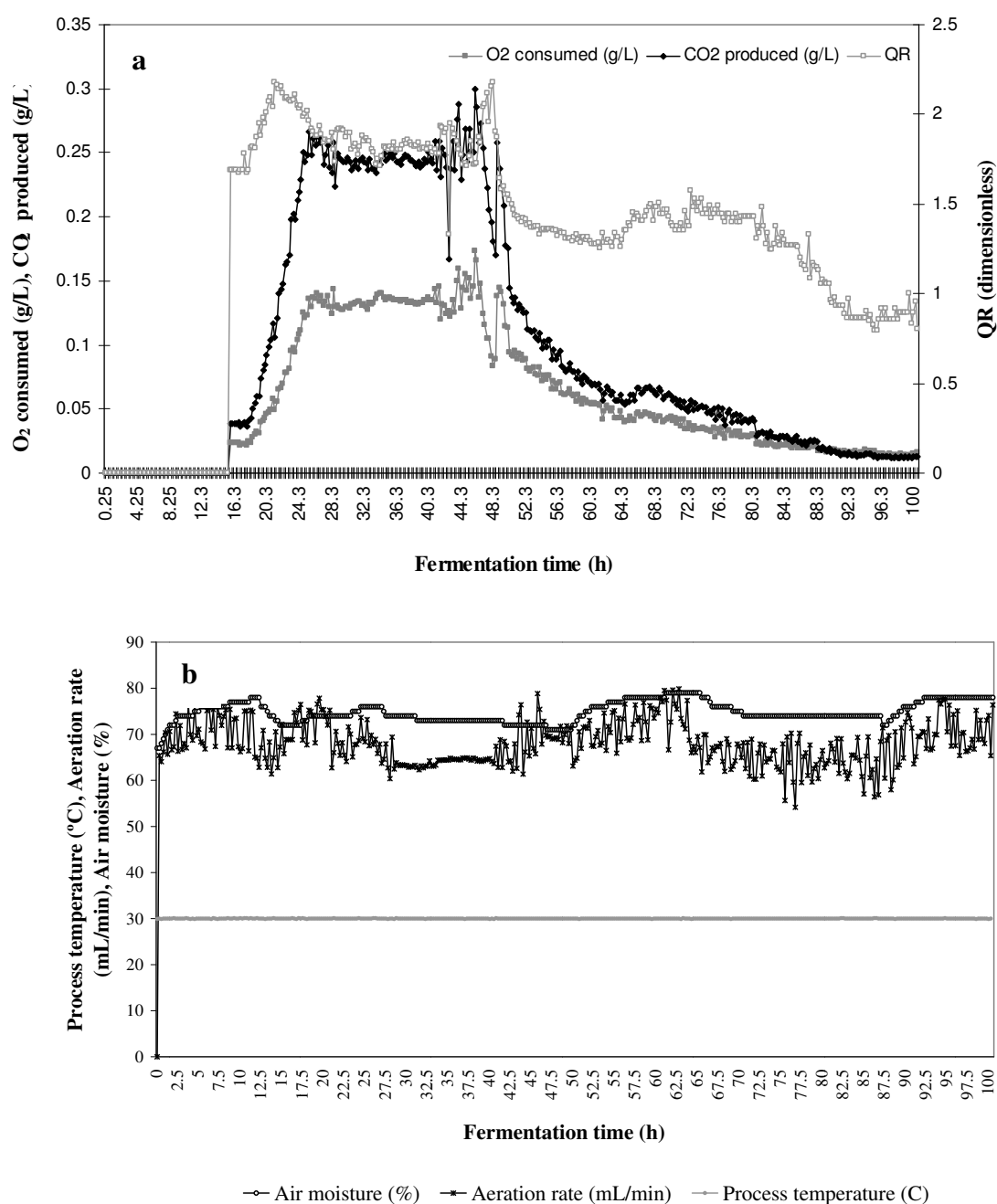


Fig. 3 - a) Results of O₂ consumption, production of CO₂ (Linear correlation: 0.988), and respiratory quotient Q in the course of fermentation time. b) Data obtained during process monitoring. Process temperature (°C), air moisture (%) and aeration rate (mL/min).

3.3 Correlation biomass-phytase

A correlation analysis (not shown) of the data from whole columns (biomass and pigments produced) presented in **Table 4**, showed the correlation coefficient $R^2 = 0.9863$, which suggested a fair proportionality between both factors. Therefore a

correlation for biomass concentration x phytase activity could be used to estimate biomass in further fermentations (**Fig.5**).

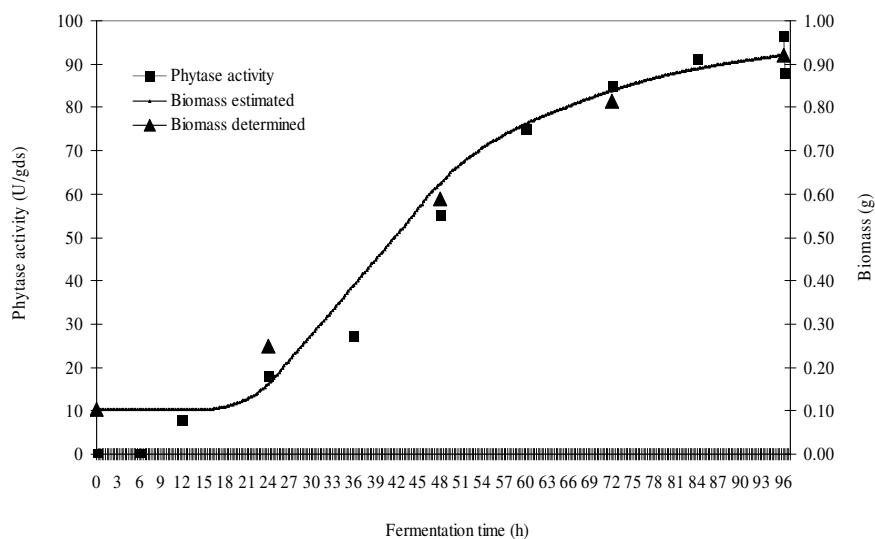


Fig. 4 - Determined biomass, estimated biomass and phytase production in SSF by *A. niger* FS3

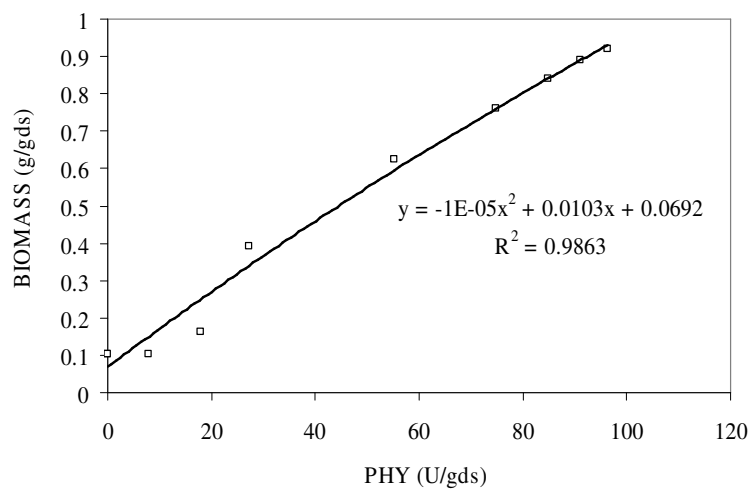


Fig. 5 - Plot of phytase activity versus biomass for correlation analysis

The following equation was defined for biomass estimation:

$$\text{BIOMASS} = -1^{-05}\text{PHY}^2 + 0.0103\text{PHY} + 0.0692, R^2 = 0.9863$$

where BIOMASS: biomass in the dry fermented citric pulp (g/g), PHY: phytase activity, in U/gds. (Carvalho et al 2006) also correlated pigment production with biomass during the course of fermentation, as an analysis alternative for more simple than ergosterol extraction and chromatography analysis. Whereas this expression should be used with care, since it probably depends on the microorganism used and on the cultivation conditions.

Table 4 - Biomass and enzyme production by dry fermented substrate at different fermentations stages – data used for correlation

Time (h)	Phytase activity (U/gds)	Estimated biomass (g/gds)
0	0.00	0.1027
6	0.00	0.1027
12	7.91	0.1027
24	17.93	0.162
36	27.35	0.3908
48	55.24	0.623
60	74.94	0.7624
72	84.88	0.8394
84	91.15	0.8897
96	96.23	0.9208

Correlation: 0.993

Table 4 represents a material balance on a column. From the values of time, biomass and consumed O₂ were obtained the following values using the program FERSOL2: $Y_{X/O} = 0.144$ g biomass/g O₂, maintenance coefficient (m) = 0.08 h⁻¹ and specific growth rate (μ)=0.038h⁻¹ in the range of 0-96 hours, with a determination coefficient (R²)=0.899 that indicated good correlation between the data and the model. From FERSOL2 the same data were generated using different range of fermentation time to evaluate the same parameters. The range of 0-48 h and 48-96 h was defined because log phase was identified within 48 hours.

Table 5 - Estimations of yield coefficient, specific growth rate and maintenance coefficient through the program FERSOL2

Process Parameters	0 to 96 hours	0 to 48 hours	48 to 96 hours
Yield coefficient ($Y_{x/o}$)	0.144	0.146	0.16
Specific growth rate (μ) (h^{-1})	0.032	0.053	0.0075
Maintenance coefficient (m)	0.080	0.16	0.145
Determination coefficient (R^2)	0.899	0.945	0.951

Describing microbial dynamics is of great importance for many different applications (Bodegon 2007). So, parameters as described in Table 5 are important to understand growth performance and phytase production during citric pulp fermentation with forced air. In the range between 48 to 96 hours, a high maintenance coefficient was observed compared to the value from 0 to 48 hours.

Maintenance coefficient (m) represents the energy consumed for functions other than the production of new cell material (Pirt 1965). The nongrowth components thus included and determined empirically when measuring maintenance are (1) shifts in metabolic pathways, (2) energy spilling reactions, (3) cell motility, (4) changes in stored polymeric carbon, (5) osmoregulation, (6) extracellular losses of compounds not involved in osmoregulation, (7) proofreading, synthesis and turnover of macromolecular compounds such as enzymes and RNA, and (8) defense against O_2 stress (Bodegon 2007, Russel 1995, Mason et al 1986, Stouthamer et al 1990). Some reasons may be considered as effect of a high maintenance coefficient after 48 hours of fermentation. First, aeration could not be sufficient to aerobic fermentation, but this factor was optimized in a factorial design as showed above indicating that high aeration rate does not influence in increasing phytase production. Another point could be the compaction of fermented substrate in the column, which induces the obstruction of the air inlet. If biomass does not have enough oxygen to aerobic fermentation, an alteration in fungal metabolism may be presumable.

Table 6 - Values of time, biomass and pigment produced, consumed O₂ and yield of biomass from substrate (Y_{X/S}), yield of product from substrate (Y_{P/S}), conversion of oxygen in biomass (Y_{X/O}) and oxygen in product (Y_{P/O}) used for the estimations of kinetic parameters through the program FERSOL2

Fermentation time (h)	Produced biomass (g/gds)	Total sugars (g/column)	Produced biomass (g/column)	Phytase activity (U/gds)	Phytase produced (U/column)	Phy/Bio	O₂ consumed (g/h)	O₂ acum.	Y_{P/S} (U/g)	Y_{X/S} (g/g)	Y_{P/O} (g/g)	Y_{X/O} (g/g)	Y_{P/X} (U/g)
0	0.1027	24.63	2.42	0.00	0.00	0	0.00	0.00	-	-	-	-	-
24	0.2495	22.01	5.72	17.93	1219.24	213.15	0.108	1.74	465.36	1.26	700.71	1.89	369.5
48	0.5893	15.16	13.29	55.24	3756.32	282.64	0.14	14.49	370.37	1.10	198.98	0.59	335.1
72	0.8152	11.77	18.12	84.88	5771.84	318.53	0.034	20.47	594.55	1.42	891.82	0.81	417.3
96	0.9216	7.71	20.16	96.23	6543.64	324.58	0.014	22.73	190.09	0.50	207.55	0.90	378.3

The yield of product from substrate ($Y_{P/S}$) showed a peak at 72 hours. It means that each gram of substrate consumed generated 594.55 U in the column at this fermentation time. The same happened with yield of biomass from substrate ($Y_{X/S}$), where 1.42 g of fungal biomass were produced for each g of substrate consumed at 72 hours of fermentation, since phytase and biomass are correlated.

4. Conclusions

The results of 32 factorial design indicates higher level of phytase production at 65% of initial moisture and air flow at 1.0 Nml/g.min i.e. Phytase synthesis is encouraged with forced air in column type bioreactor. This work also showed a simple alternative for biomass determination in citric pulp fermentation by *A. niger* FS3 than ergosterol extraction and chromatography analysis. The program FERSOL2 estimated process parameters as yield of biomass from oxygen ($Y_{X/O}$), maintenance coefficient (m) and specific growth rate (μ) showing a good correlation between the data and the model.

References

- Greiner R, Konietzny U (2006), Phytase for Food Application, Food Technology Biotechnology 44 (2) 125–140.
- Soccol CR, Silva ARZ, Rodriguez-Leon JA, Germano S. Produção de Fitase por Fermentação no Estado Sólido utilizando Produtos, Subprodutos e Resíduos Agroindustriais para Aplicação na Alimentação Animal. INPI Patente N. PI0203596 (29 ago. 2002).
- Greiner R (2007), Inositol Phosphates: Linking Agriculture and the Environment. Phytate-degrading enzymes: regulation of synthesis in microorganisms and plants (3) 78-95.
- Pandey A, Szakacs G, Soccol R, Rodriguez-Leon JA, Soccol VT (2001), Production, purification and properties of microbial phytases, Bioresource Technology 77, 203–214.

Mitchell DB, Vogel K, Weimann BJ, Pasamontes L, Loon APGM (1997), Microbiology, 143, 245–252.

Sandberg AS, Andersson H (1988), Effect of dietary phytase on the digestion of phytate in the stomach and small intestine of humans, Journal of Nutrition, 118(4), 469-473.

Hurrell RF (2004), Phytic acid degradation as a means of improving iron absorption. International J for Vitamin and Nutrition Research 74 (6), 445–452.

Mitchell, C. ABC Science. Urine offers rich phosphorus sources. Discovery News. 10 jul. 2007 <<http://dsc.discovery.com/news>>

Ramachandran S, Singh SK, Larroche C, Soccol CR, Pandey A.(2007), Oil cakes and their biotechnological applications. A review. Bioresource Technology 98, 2000–2009

Pandey A, Soccol CR (1998), Bioconversion of biomass: A case study of ligno-cellulosics bioconversions in solid state fermentation. Brazilian Archives Biology Technology, 41(4), 379-390.

Pandey A (1994), Solid state fermentation- an overview. In- Solid State Fermentation, A. Pandey (ed.), Wiley Eastern Publishers, New Delhi, 3-10.

Pandey A, Soccol CR, Mitchell D (2000a), New developments in solid state fermentation: I bioprocesses and products. Process Biochemistry 35, 1153-1169.

Pandey A, Soccol CR, Mitchell D (2000b), New developments in solid state fermentation: I- bioprocesses and products. Process Biochemistry 35, 1153–1169.

Pandey A, Soccol CR, Nigam P, Soccol VT (1999), Biotechnological potential of agro-industrial residues: I Sugarcane bagasse. Bioresource Technology 74, 69-80.

Pandey A. (1992), Recent process developments in solid state fermentation, Process Biochemistry 27, 109-116.

Macedo G, Fraga L (2007), Production of cutinase by *Fusarium oxysporum* in solid-state fermentation using agro-industrial residues. Abstracts / Journal of Biotechnology 131S, S211–S241

Soccol CR, Vandenberghe LPS (2003), Overview of applied solid-state fermentation in Brazil. Biochemical Engineering Journal, 13(2-3), 205-218.

Pandey A, Selvakumar P, Soccol CR, Nigam P (1999), Solid-state fermentation for the production of industrial enzymes, Current Science (77) 149–162.

Moo-Young M, Moreira AR, Tengerdy RP (1983), Principles of solid substrate cultivation, in: J.E. Smith, D.R. Berry, B. Kristiansen (Eds.), Filamentous Fungi, (2), Edward Arnold, London, 557–566.

Doelle HW, Mitchell DA, Rolz CE (1992), Solid substrate cultivation. London: Elsevier Applied Science, v.1, 454 p.

Raimbault M (1981), Travaux et Documents de l'Orstom, n. 27, París.

Okasaki N, Sugama S, Tanaka T (1980), Mathematical model of surface culture of koji mold. Journal Fermentation Technology 58 (5), 471.

Matcham SE, Jordan BR, Wood DA (1985), Estimation of fungal biomass in a solid substrate by three independent methods, Appl. Microbiol. Biotechnol. (21) 108–112.

Desgranges C, Vergoignan C, Georges M, Durand A (1991), Biomass estimation in solid state fermentation, Applied Microbiology Biotechnology (35) 200–205.

Carvalho JC, Pandey A, Oishi, BO, Brand D, Rodriguez-León JA, Soccol CR (2006), Relation between growth, respirometric analysis and biopigments production from *Monascus* by solid-state fermentation. Biochemical Engineering Journal 29 (2006) 262–269.

Bellon-Maurel V, Orliac O, Christen P (2003), Sensors and measurements in solid state fermentation: a review. Process Biochemistry (38), 881-896.

Spier MR, Greiner UK, Greiner R, Rodríguez-León JA, Carvalho JC, Woiciechowski AL, Soccol CRO (2007), Relation between Respirometric Data and Amylolytic Enzyme Production by SSF in Column-Type Bioreactor, International Journal of Chemical Reactor Engineering 5: A67.

Nishio N, Tai T, Nagai S (1979), Hydrolase production by *Aspergillus niger* in solid state cultivation. Eur. Journal Applied Microbiology Biotechnology 8, 263.

Carrizalez V, Rodríguez H, Sardiña I (1981), Determination of the specific growth rate of molds on semi-solid cultures. Biotechnology Bioengineering 23(2), 321.

Rodríguez-León JA, Sastre L, Echevarria J, Delgado G, Bechstedt W (1988), A mathematical approach for the estimation of biomass production rate in solid-state fermentation. Acta Biotech. 8, 307-310.

Vicini G, Mitchell DA, Boit SD, Gern JC, Rosa AS, Costa RM, Dalsenter FDH, Meien OF, Krieger N (2001), Analysis of growth kinetic profiles in solid-state fermentation, Food Technology Biotechnology 39 (40), 1–23.

Hamidi-Esfahani Z, Hejazi P, Shojaosadati SA, Hoogschagen M, Vasheghani-Farahani E, Rinzema A (2007), A two-phase kinetic model for fungal growth in solid-state cultivation, Biochemical Engineering Journal (36), 100–107.

Spier MR, Greiner R, Rodríguez-León JA, Woiciechowski AL, Pandey A, Soccol VT, Soccol CR (2008), Phytase production using citric pulp and other residues of the agro-industry in SSF by fungal isolates, Food Technology Biotechnology 46 (2008), 178-182.

Prado FC, Vandenberghe LPS, Soccol CR (2005), Relation between citric acid production by solid state fermentation from cassava bagasse and respiration of *Aspergillus niger* LPB 21 in semi-pilot scale. Brazilian Archives of Biology and Technology, Curitiba, 48, 29-36.

Seitz LM, Sauer DB, Burroughs R, Mohr HE, Hubbard JD (1979), Ergosterol as a measure of fungal growth. Phytopathology 69, 1202-1203.

Sturm W, Soccol CR, Dergint DE, Rodriguez-Leon JA, Magalhaes DCNV, Pandey A (2007), Informatics in Solid-State Fermentation (Chapter 08). In: Ashok Pandey; Carlos Ricardo Soccol; Christian Larroche. (Org.). Current Developments in Solid-State Fermentation. 1 ed, v. 1, 169-180. New York: Springer Science + Business Media, LLC, 2007.

Pandey A, Soccol CR, Rodríguez-león JA, Nigam P (2001), Solid State Fermentation in Biotechnology: Fundamentals and Applications. Asiatech Publishers Inc. New Dehli India.

Heinonen JK, Lahti RJ (1981), A new and convenient colorimetric determination of inorganic orthophosphate and its application to the assay of inorganic pyrophosphatase, Analytical Biochem. 113, 313-317.

Oostra J, Comte EP, van den Heuvel JC, Tramper J, Rinzema A (2001), Oxygen diffusion limitation in solid-state fermentation, Biotechnology Bioengineering 74, 13-24.

Raghavarao KSMS, Ranganathan TV, Karanth NG (2003), Some engineering aspects of solid-state fermentation. Biochemical Engineering Journal 13, 127–135.

Singhania RR, Soccol CR, Pandey A (2008), Recent advances in Solid-state Fermentation, Biochemical Engineering Journal, doi:10.1016/j.bej.2008.10.019.

Krishna C, Nokes SE (2001), Influence of inoculum size on phytase production and growth in solid-state fermentation by *Aspergillus niger*. Transactions of the ASAE (44) 4, 1031-1036.

Greiner R (2005), Current biochemical research on phytase genes in microorganisms and plants. Inositol Phosphates in the Soil-Plant-Animal System. Bouyoucos Conference. USA.

Shieh TR, Wodzinski RJ, Ware JH (1969), Regulation of the formation of acid phosphatase by inorganic phosphate in *Aspergillus ficuum*. Journal Bacteriology 100, 1161-1165.

Vassilev N, Vassileva M, Bravo V, Fernández-Serrano M, Nikolaeva I (2007), Simultaneous phytase production and rock phosphate solubilization by *Aspergillus niger* grown on dry olive wastes. Industrial Crops and Products 25 (3), 332-336.

Singh B, Satyanarayana T (2007), Phytase production by a thermophilic mould *Sporotrichum thermophile* in solid state fermentation and its potential applications. *Bioresource Technology* 99(8), 2824-2830.

Roopesh K, Ramachandran S, Nampoothiri KM, Szakacs G, Pandey A (2006), Comparison of phytase production on wheat bran and oilcakes in solid-state fermentation by *Mucor racemosus*, *Bioresource Technology* 97, 506–511.

Chantasartasamee K, Ayuthaya DI, Intarareugsorn S, Dharmsthiti S (2005), Phytase activity from *Aspergillus oryzae* AK9 cultivated on solid state soybean meal medium. *Process Biochemistry* 40 (7) 2285-2289.

Fujita J, Shigeta S, Yamane YI, Fukuda H, Kizaki Y, Wakabayashi S, Ono K (2003), Production of two types of phytase from *Aspergillus oryzae* during industrial koji making. *Journal of Bioscience Bioengineering* 95(5), 460-465.

Gautam P, Sabu A, Pandey A, Szakacs G, Soccol CR (2002), Microbial production of extra-cellular phytase using polystyrene as inert solid support. *Bioresource Technology*, 83(3), 229-233.

Sabu A, Sarita S, Pandey A, Bogar B, Szakacs G, Soccol CR (2002), Solid-State Fermentation for Production of Phytase by *Rhizopus oligosporus*, *Applied Biochemistry and Biotechnology* 103, 251-260.

Papagianni M, Nokes SE, Filer K (1999), Production of phytase by *Aspergillus niger* in submerged and solid-state fermentation. *Process Biochemistry* 35(3-4), 397-402.

Ebune A, Al-Asheh S, Duvnjak Z (1995), Production of phytase during solid state fermentation using *Aspergillus ficuum* NRRL 3135 in canola meal. *Bioresource Technology* (53), 7-12.

Bogar B, Szakacs G, Linden JC, Pandey A, Tengerdy RP (2003), Optimization of phytase production by solid substrate fermentation, *Journal of Industrial Microbiology and Biotechnology* 30 (3), 183-189

El-Batal AI, Karem HA (2001), Phytase production and phytic acid reduction in rapeseed meal by *Aspergillus niger* during solid state fermentation. Food Research International 34(8), 715-720.

Bodegom P (2007), Microbial Maintenance: A Critical Review on Its Quantification, Microb Ecol. 2007 53(4): 513–523. doi: 10.1007/s00248-006-9049-5.

Pirt SJ (1965), The maintenance energy of bacteria in growing cultures. Proc R Soc London B 163: 224–231

Russell JB, Cook GM (1995), Energetics of bacterial growth: balance of anabolic and catabolic reactions. Microbiol Rev 59, 48–62.

Mason, CA, Hamer, G, Bryers, JD (1986) The death and lysis of microorganisms in environmental processes. FEMS Microbiol Rev 39: 373–401.

Stouthamer AH, Bulthuis BA, van Verseveld HW (1990), Energetics of growth at low growth rates and its relevance for the maintenance concept. In: Poole, RK, Bazin, MJ,

CHAPTER IV

IMPROVEMENT OF PHYTASE ADSORPTION AND RECOVERY BY OPTIMIZATION OF QUICK BATCH PROCEDURES

Abstract

The identification of suitable conditions for the first step of phytase purification was performed in batch. There is a lack of data in literature describing the influence of different conditions in the improvement of the adsorption and yield in enzyme purification using crude extract from SSF ignoring its composition. Buffer salt, pH buffer and crude extract ionic strength were the variables tested to improve binding of enzyme to cation exchange resins CM and SP-sepharose. The results showed that parameters established as SP-sepharose FF in glycine-HCl pH 2.85 and enzyme diluted in the same buffer in the proportion 1:3 (v/v), resulted in a substantial recovery of enzyme (59.61%) compared to the recovery percentage in CM-sepharose (56.61%) considering the study conducted in tubes instead of columns. This batch study is a fast way to define the previous parameters to run ion exchange chromatography.

Key words: phytase, adsorption, recovery, ion exchange chromatography

1. Introduction

Phytases (*myo*-inositol hexakisphosphate phosphohydrolases; EC 3.1.3.8 and EC 3.1.3.26) are enzymes capable of hydrolyzing phytate (*myo*-inositol (1,2,3,4,5,6) hexakisphosphate) (Mitchell et al., 1997; Pandey et al., 2001) and contributes to improve mineral bioavailability (Sandberg and Andersson, 1988). Phytate has a strong affinity to chelate proteins, starches, metals (especially iron, zinc, calcium and magnesium). The chelates thus formed, increase the amount of insoluble salts of the above minerals, which have poor absorption characteristics and hence low bioavailability (Hurrell, 2004). Low absorption of these nutrients from cereals is considered a factor in the aetiology of mineral deficiencies. Without an adequate supply of bioavailable iron, mainly the infants soon become iron deficient (Frontela, 2008). Iron deficiency may induce complications such as anaemia, decrease in psychomotor and mental development, and reduction in immune system (Hurrell, 2004). Low calcium bioavailability affects the attainment of peak bone mass, which may increase the risk of fractures and osteoporosis in later life (Frank, 2006). Zinc deficiency can lead to an increased severity and incidence of diarrhoea and respiratory infections, as well as decreased growth rates (Harland and Oberleas, 1987).

Before phytase can be use in food and feed the properties of pure enzymes must be determined. Suitable conditions value for column chromatography such as type of cationic exchanger, buffer, ionic strength of enzyme solution and pH could be elucidated by batch.

2. Materials and Methods

2.1 Phytase production in SSF

Experiments were conducted in 250 mL Erlenmeyer flasks containing citric pulp (CP), 33 mM citrate buffer pH 5.0 to adjust moisture level to 60%, inoculated with *Aspergillus niger* FS3 and incubated at 30°C for 96 hours according to Spier et al. (2008).

2.2 Crude enzyme extraction

30 mM citrate buffer (pH 5.0) (1:10 w/v) was used to extract phytase from fermented citric pulp. The suspension was clarified by filtration through cheesecloth. The filtrate obtained was centrifuged (5000 rpm/20 min) to remove particles, cells and spores. The clarified supernatant was used for batch studies and the phytase activity was determined

using a modified method of Heinonen and Lahti (1981) as described by Greiner et al. (1997). All experiments were performed in triplicate and average values were reported.

2.3 Identification of suitable conditions for cationic exchange chromatography

Batch studies were performed to identify suitable conditions for cationic exchange chromatography (Figure 3). Buffer salt, pH buffer and crude extract dilution were the variables tested to improve binding of enzyme to cation exchange resins CM-Sepharose FF and SP-Sepharose FF (Pharmacia Biotech, Sweden). Crude phytase solution was diluted 1:2 (v/v) and 1:3 (v/v) with two buffers: 25 mM glycine-HCl pH 2.85 and 10 mM Na-acetate pH 4.3, respectively, following the strategy used by Ullah and Gibson (1987). Then, phytase activity was determined (A = initial activity). CM-sepharose (weak cation exchanger) and SP-sepharose (strong cation exchanger) (Pharmacia Biotech, Sweden) were distributed equally to 50 mL centrifuge tubes. Four cationic chromatography steps were performed: equilibration, loading, washing, and elution (**Fig. 1**).

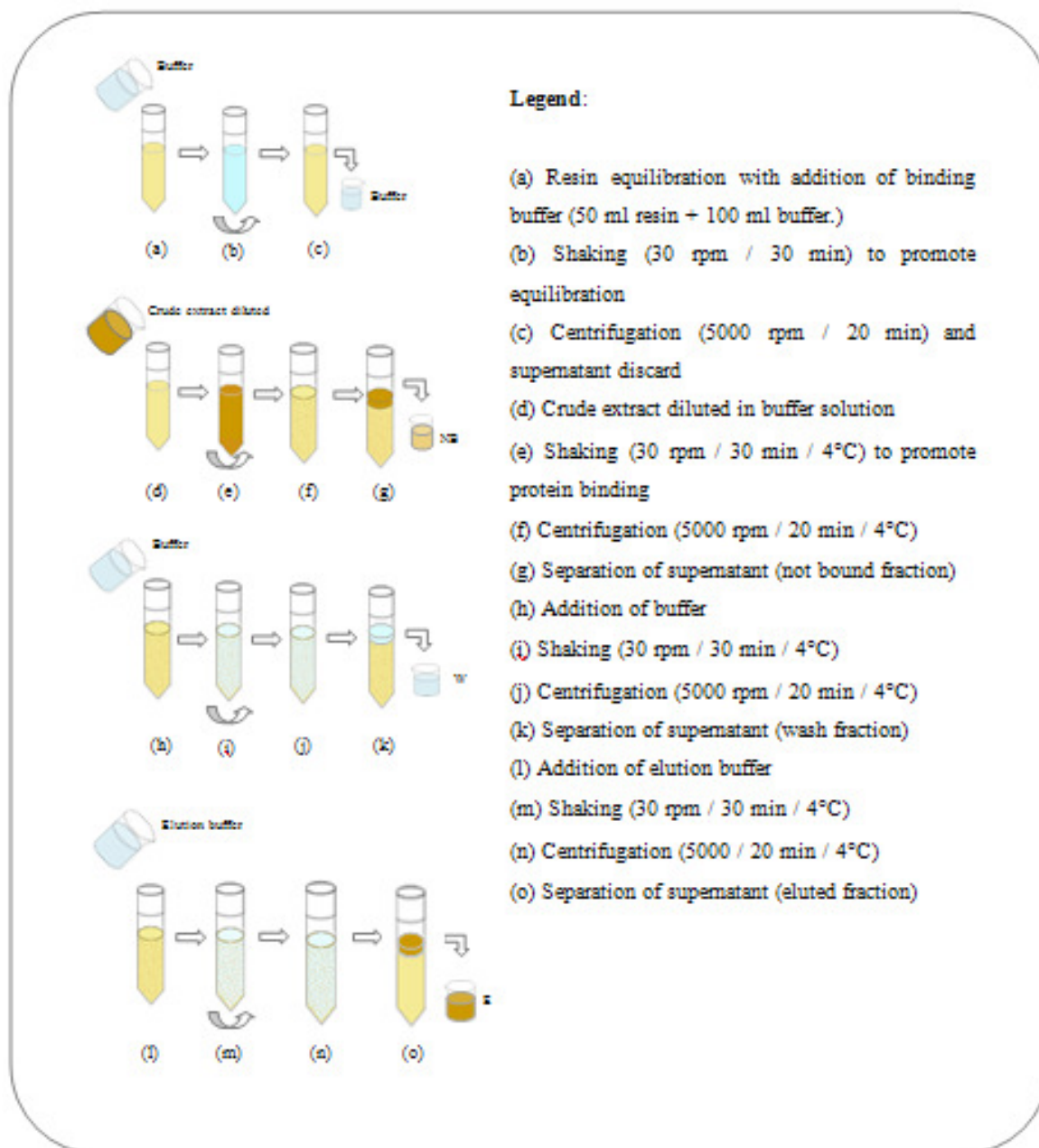


Fig. 1 - Modified ion exchange procedure showed in this work – Quick batch procedure

2.4 Equilibration

The first step consisted of equilibrating the CM and SP resins in buffer (25 mM glycine-HCl pH 2.85 and 10 mM Na-acetate pH 4.3, respectively), in the proportion resin/buffer 1:1 (v/v) gently agitated (30 rpm / 30 min at room temperature). Then the test tubes were centrifuged (5,000 rpm / 20 min) and this step was repeated twice. The supernatants were discarded.

2.5 Load

The tubes containing equilibrated resins were completed with 50 mL of each diluted crude phytase. The mixture was gently agitated (30 rpm / 30 min / 4°C) to induce the positive character of protein to adsorb in a negative charged resin. Then, the tubes were centrifuged in the same conditions described above. The supernatants were transferred to new centrifuge tubes and named NB and used for phytase activity determination (NB = phytase activity not bounded to the exchange material).

2.6 Wash

50 mL of 25 mM glycine-HCl pH 2.85 and 10 mM Na-acetate pH 4.3, respectively, were added to the CM and to the remaining SP material (resin+enzyme) from load step. After incubation for 30 min at 30 rpm and 4°C the test tubes were gently agitated and centrifuged at 5,000 rpm / 20 min / 4°C, then the supernatants were transferred to new assay test tubes and used for phytase activity determination (W = phytase activity in the wash solution).

2.7 Elution

The Enzyme bonded to the column material was eluted with 50 mL of 1M NaCl in the corresponding buffer. After incubation under gentle shaking for 30 min, 30 rpm and 4°C, the test tubes were centrifuged at 5,000 rpm / 20 min / 4°C. Supernatants were transferred to new assay tubes and then used for phytase activity determination (E = eluted phytase activity). Finally, the column material was cleaned according to the supplier instructions and stored in refrigerator for further use.

2.8 Protein determination

Protein concentration was determined photometrically using BSA as standard-solution according to Bradford method (Bradford, 1976).

Bond (B) and eluted (E) phytase activity were calculated according to equations (1) and (2):

$$B [\%] = [A - (NB + E)] \times 100 / A \quad (1)$$

$$E [\%] = E \times 100 / A \quad (2)$$

2.9 Cationic exchange chromatography (step 1)

The first step of phytase purification from crude extract was conducted at 4°C room temperature, using a glass column (35 mm x 300 mm) carried with SP Sepharose Fast Flow resin (Pharmacia Biotech, Sweden), a strong cation exchanger, which has ion exchange sulphopropyl group ($-\text{O}-\text{CH}_2\text{CHOHCH}_2\text{OCH}_2\text{CH}_2\text{CH}_2\text{SO}^{-3}$). The column was pre-equilibrated with 25 mM glycine-HCL (pH 2.85) and loaded with 100 mL of crude phytase extract diluted 1:3 with 25mM glycine-HCl pH 2.85. Later the column was washed with the same buffer to remove proteins and others positive charged compounds not adsorbed in the column. A gradient former was coupled in the column to promote a linear gradient of NaCl to cause the elution of proteins according to their different characteristics of elution. This experiment was performed at a 1mL/min flow rate. Fractions (10 mL) were collected in a SuperFrac fraction collector (GE Healthcare, Sweden) and assayed for phytase activity. The higher phytase activity fractions were pooled and dialyzed against 50 mM Tris-HCl. Finally, the column was cleaned according to the supplier instructions and kept in 0.0001% NaN_3 solution (**Fig. 2**).

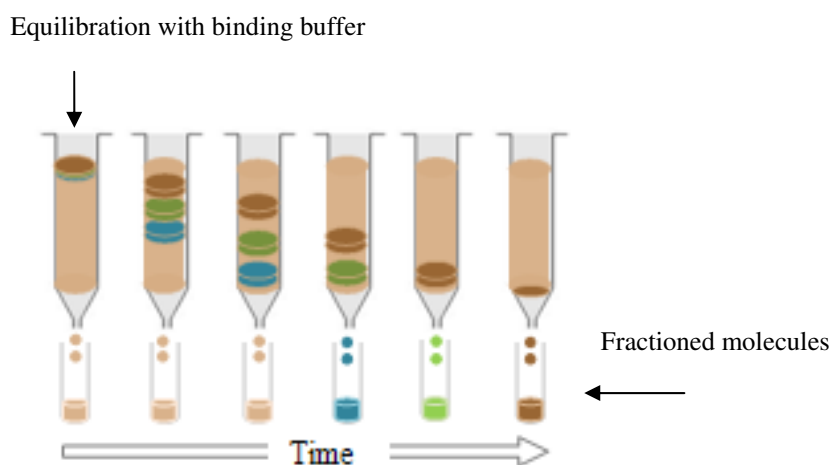


Fig. 2 - Traditional ion exchange chromatography

2.10 SDS-PAGE electrophoresis

SDS-PAGE (sodium dodecyl sulphate polyacrylamide gel electrophoresis) analysis was carried out according to Laemmli method (Laemmli, 1970). Gels were stained with Coomassie brilliant blue R-250.

3. Results and Discussion

3.1 Batch procedures with cationic exchange resins

A summary result of batch studies using CM and SP is presented in **Table 1**. In the conditions tested, the recovery percentage was lower when Na-acetate buffer pH 4.3 was applied in crude phytase dilution of 1:2. It can be inferred that the ionic strength of the crude extract is too high to allow considerable binding of phytase to cation exchange material compared to the dilution 1:3. Reducing the ionic strength of enzyme solution through dilution resulted in an improved phytase binding in the two resins used CM and SP-sepharose. Two conditions (glycine-HCL buffer/pH 2.85/crude extract dilution 1:3) had a synergistic effect on phytase recovery (56.61% in CM and 59.61% in SP-sepharose).

Table 1 - Results of batch studies to identify suitable conditions in CM, SP and DEAE-sepharose cationic chromatography

Buffer	Dilution of crude extract	Step	CM-Sepharose		SP-Sepharose	
			Enzyme activity* (U/mL)	(%)	Enzyme activity* (U/mL)	(%)
Na-acetate pH 4.3	1:2	A	1.18	-	1.20	-
		B	0.30	25.6	0.59	47.1
		E	0.09	8.3	0.47	44.3
	1:3	A	1.02	-	1.04	-
		B	0.38	37.5	0.50	48.7
		E	0.15	15.2	0.47	45.7
Glycine-HCl pH 2.85	1:2	A	1.18	-	1.18	-
		B	0.30	25.8	0.58	49.5
		E	0.08	6.8	0.54	45.8
	1:3	A	1.02	-	1.04	-
		B	0.78	37.5	0.65	62.0
		E	0.58	56.6	0.62	59.6

A (initial phytase activity); B (bonded phytase activity); E (eluted phytase activity);*average

The strong cationic exchanger induced adsorption improvement when parameters stabilised SP-sepharose FF, glycine-HCl pH 2.85, and enzyme dilution of 1:3 (v/v) were applied. The study in tubes achieved 62.07% of phytase bonded and 59.61% of initial phytase

activity was recovery by 1M NaCl elution step. This batch study is an easy and fast way to define the previously parameters to run ionic chromatography. Partial phytase purification profile in SP-sepharose column (35 mm x 300 mm) is showed in **Fig.3**.

Fifty one fractions were collected at a flow rate of 1 mL/min. Fractions 1 to 10 did not present protein content. Phytase activity was assayed in all fractions. The results of phytase activity, OD 280nm and NaCl gradient are reported in Figure 1. Phytase was eluted since 0.25M NaCl when a 260 mL volume was eluted. It was evident that the elution of 4 peaks of proteins, where the second peak corresponds to maximum phytase activity detected in this chromatography. Fractions 27-36 totalizing 100 mL were pooled and prepared to the next step of purification (data not shown).

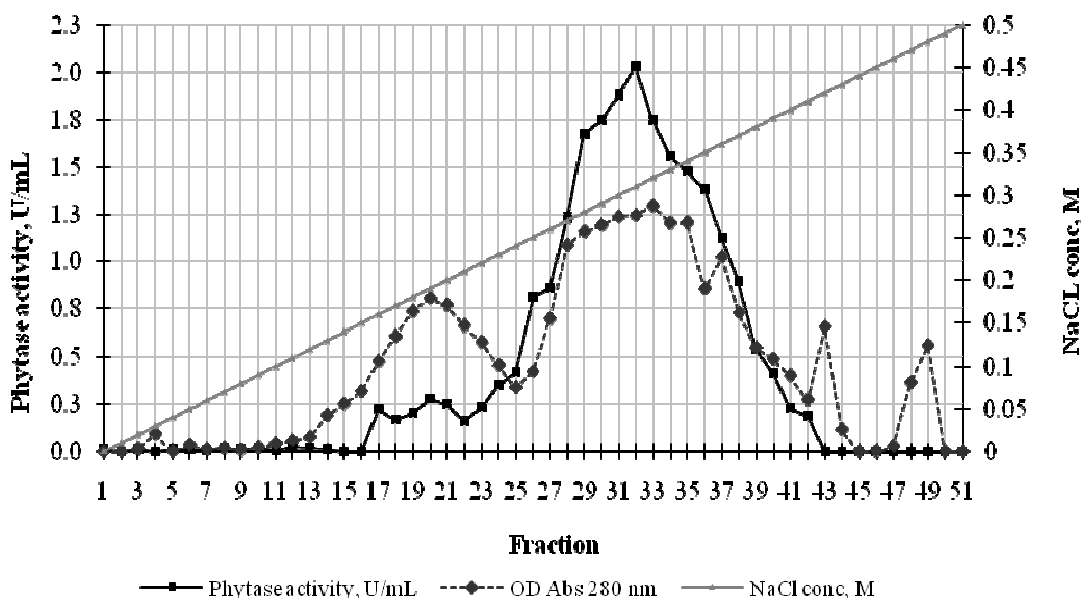


Fig. 3 - Cationic chromatography in SP sepharose FF column showing peaks of phytase activity, proteins and linear gradient.

In SP-sepharose column, phytase was purified 5.6-fold from the crude phytase diluted with 54.54% recovery.

3.2 SDS-PAGE electrophoresis

SDS-PAGE (**Fig. 4**) shows a partial purification of phytase. Compared to crude enzyme solution, which presented a high protein concentration (lane 1 and 2), the lane 3 represents phytase pooled from SP-sepharose cationic chromatography column. SDS-PAGE gel presented 16 (Lane 1) and 15 (Lane 2) bands, respectively, while after cationic exchange chromatography gel showed only 6 bands, evidencing cationic exchange chromatography effectiveness to first step of the purification process. Other steps must be applied. Suggestion to continue the work is combined anionic exchange chromatography and chromatofocusing to purify the enzyme. These steps are being studied yet.

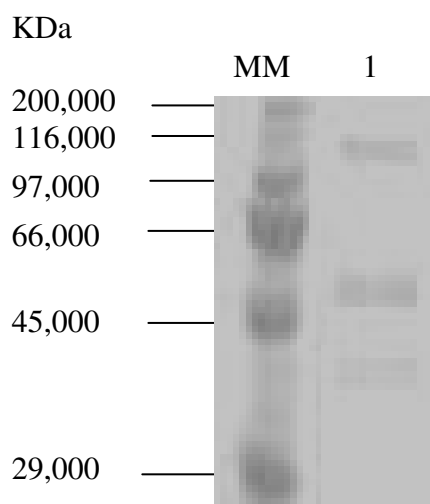


Fig. 4 - SDS-PAGE analysis of the *A. niger* FS3 phytase. MM) Molecular weight marker (SDS6H2, Sigma); 1) after SP-sepharose column (lane 1).

4. Conclusions

Batch studies demonstrated an alternative to test different parameters to perform cationic chromatography quickly before testing in the column. Although phytase from *A. niger* has previously been studied, there is a lack of data in literature comparing phytase recovery in different cationic exchange chromatography materials. Phytase has also been partially purified using the best parameters found previously in strong cationic exchanger SP-sepharose FF and SDS-PAGE electrophoresis confirmed partial purification.

References

Bradford M. *Anal. Biochem.*, 72 (1976), 248-258.

Frank R, Greer MD, Nancy F, Krebs MD (2006), The Committee on Nutrition - Optimizing bone health and calcium intakes of infants, children and adolescents. *Pediatrics*, American Academy of Pediatrics, ISSN: 1098-4275.

Frontela FJ, García-Alonso G, Ros C, Martinez (2008), Phytic acid and inositol phosphates in raw flours and infant cereals: The effect of processing. *Journal of Food Composition and Analysis* 21, 343–350.

Greiner R, Konietzny U. (1996), *Journal Biotechnology* 48:153–159.

Greiner R, Haller E, Konietzny U, Jany KD (1997), Purification and characterization of a phytase from *Klebsiella terrigena*, *Archives of Biochemistry and Biophysics* 341 (2), 201–206.

Harland BF, Oberleas D (1987), Phytate in foods. *World Review of Nutrition and Dietetics* 52, 235–259.

Heinonen JK, Lahti RJ (1981), *Anal. Biochem.* 113, 313-317.

Hurrell RF (2004), Phytic acid degradation as a means of improving iron absorption. *International J for Vitamin and Nutrition Research* 74 (6), 445–452.

Laemmli UK (1970), Cleavage of structural proteins during the assembly of the head of Bacteriophage T4. *Nature* (227), 680–685.

Mitchell DB, Vogel K, Weimann BJ, Pasamontes L, Loon APGM (1997), *Microbiology*, 143, 245–252.

Pandey A, Szakacs G, Soccol CR, Rodriguez-Leon JA, Soccol VT (2001), Production, purification and properties of microbial phytases, *Bioresource Technology* 77, 203–214.

Sandberg AS, Andersson H. (1988), *Journal of Nutrition*, 118(4), 469-473.

Spier MR, Greiner R, Rodriguez-León JA, Woiciechowski AL, Pandey A, Soccol VT, Soccol CR (2008), Phytase Production Using Citric Pulp and Other Residues of the Agro-Industry in SSF by Fungal Isolates. *Food Technoogy Biotechnology* 46 (2), 176–180.

Ullah AHJ, Gibson DM (1987), Extracellular phytase (E. C. 3. 1. 3. 8) from *Aspergillus ficuum* NRRL 3135: purification and characterization. *Prep Biochemistry* 17:63–91.

CHAPTER V

PHYTASE PRODUCED ON A NEW SUBSTRATE: PURIFICATION AND CHARACTERIZATION

Abstract

A producing phytase fungus was isolated from Brazilian soil sample and identified as *Aspergillus niger* FS3. The phytase produced for the first time in citric pulp bran was purified and characterized. Citric pulp is an agro industrial residue from citrus processing industry, containing low inorganic-P content. The phytase was purified by electrophoretic homogeneity by cationic-exchange, anionic-exchange chromatography and cromatofocusing steps. On SDS–PAGE analysis, the molecular weight of the purified phytase was calculated to be approximately 100 kDa. The phytase has an optimum pH of 5.0-5.5 and an optimum temperature of 60°C. The phytase displayed high affinity for phytate and the K_m was 0.52 mM.

Key words: phytase, *Aspergillus*, solid-state fermentation, citric pulp, purification.

1. Introduction

Brazil is the main producer of orange juice in the world, exporting 1,271 million of tons of this commodity freezed and concentrated (IBGE 2007, Abecitrus 2008). The production in 2008 was 9.5% higher than the year before (IBGE 2008). During citrus juice industrial production, citric pulp bran is the main residue generated (Nelson and Tressler 1980, Spier et al 2008) composed basically of the citrus fruit bagasse, rags and seeds equivalent to more or less 50% of the processed fruit mass (Abecitrus 2008, Citroex 2008). This residue is pressed and dried achieving final moisture content of 12% (Abecitrus 2008). The composition of citric pulp bran is : proteins 5.5-6.2%; carbohydrates 70% (pectin and residual sugars); crude fiber 14%; lipids 1.2-1.3% and minerals matter < 8% (Abecitrus 2008, Citroex 2008). Among these compounds, the low inorganic-P content is one of characteristics that differ from others residues used to phytase production. Most of the phytase studies (Tang et al 2008, Sing and Satyanarayana 2008, Roopesh et al 2006, Fujita et al 2003, Casey and Walsh 2003, Quant et al 2004, Li et al 2008) use substrates without mention the inorganic-P content of these raw materials. There is a lack of data in the literature using citric pulp to produce phytase or even other residue with low inorganic-P content. Low inorganic-P content stimulates phytase synthesis (Spier et al 2008). Besides, inorganic-P in concentration above 5 µg/mL is well known to repress phytase synthesis in many fungi (Vats and Banerjee 2002, Quan et al 2001). On the other hand, low inorganic-P content induces phytase synthesis. Citric pulp bran shows low inorganic-P content (0.025 µmol per gram of citric pulp) when compared to others agro industrial residues such as wheat bran, soy bran, rice bran and apple pulp (Spier et al 2008). These characteristics permit the use of citric pulp bran to produce phytase and study its purification, characterization and stability properties.

The present work aim to study the purification and the properties of a phytase produced by a new fungus strain in citric pulp bran fermentation, considering that this residue was not applied in biotechnology processes yet and it shows economic viability to produce interesting biomolecules such as phytase using this low cost industrial residue.

2. Materials and Methods

2.1 *Micro-organism and fermentation procedures*

A. niger FS3 was isolated from soil samples collected at the Northeast of Parana State (Brazil) according to the technique described by Spier et al (2008). Phytase was generated during solid-state fermentation using 0.8-2.0mm particles of citric pulp (CP) from Cargill (Brazil) into 250mL flasks by the *A. niger* FS3 isolated Spier et al 2008. The medium consisted of 0.33M Na-citrate pH=5.0 buffer containing: ammonium citrate 0.5%, KCl 2mM, MgSO₄·7H₂O 5 mM, ZnSO₄·7H₂O 5mM to adjust the initial moisture at 65%. The medium was inoculated with a spore suspension of *A. niger* FS3 10% (w/v) related to the citric pulp solid media. The inoculated media was thoroughly mixed and placed into the columns. The columns were incubated at 30°C in a water bath during 96 hours with aeration of 1.2 Nml/g.min to perform the fermentation process. Extracellular crude phytase was extracted with an initial filtration through Whatman n°1 filter paper and the filtrate was centrifuged (4500 g / 15min / 4°C). The supernatant was stored at 4°C until the purification procedures.

2.2 *Protein determination*

Protein concentration was photometrically measured using BSA (bovine serum albumin) against standard-solution curve according to Bradford method (Bradford 1976).

2.3 *Enzyme assay*

Phytase activity was determined measuring the amount of inorganic phosphate liberated during the reaction with the crude extract and phytate, according to the modified method of Heinonen and Lahti (Heinonen and Lahti, 1981). 350 µL of the substrate (2.5mM sodium phytate-Sigma in 0.2M sodium acetate buffer, pH 4.5 and 50 µL of the enzyme were incubated at 50°C for 30 minutes. The reaction was stopped with the addition of 1.5 mL of AAM-solution (10 mM ammonium molybdate, 5N sulphuric acid and acetone in the proportion 1:1:2) and 100 µL of citric acid. One unit of the enzyme activity was defined as one µmol of inorganic phosphate released per minute per mL of enzyme solution under the defined reaction conditions.

2.4 Cationic exchange chromatography CEC

The first step of phytase purification from crude extract was conducted under normal pressure using a glass column (35 mm x 300 mm) with SP sepharoseTM FF (GE, Sweden) and pre-equilibrated with 25 mM glycine-HCl (pH 2.85). The column was loaded with crude extract containing phytase diluted 1:3 with 25mM glycine-HCl pH 2.85, and then washed with the same buffer. The proteins bounden onto the SP sepharose were eluted with a linear gradient from 0 to 0.5 M NaCl in the buffer described above and fraction with 2 mL were collected. The fractions with high phytase activity were taken, pooled and dialyzed against 50 mM Tris-HCl (pH 7.8).

2.5 Anionic exchange chromatography (AEC)

The second step was conducted using a Mono Q[®] HR 5/5, a strong anion-exchange column (Pharmacia Biotech) using 50 mM Tris-HCl buffer (pH 7.8) to equilibration. The chromatography was performed at a FPLC system with a flow rate of 1.0 mL/min. The fractions obtained in step 1 with high phytase activity were loaded into the Mono Q column and eluted using a linear gradient from 0 to 0.5 M NaCl concentration obtained with a gradient maker and stirrer using the following procedure: From 0 to 90 min, the concentration of NaCl increased from 0 to 50% of 1M NaCl (when the fractions were collected). From 90 to 110 min, the NaCl concentration increased to 100% with a flow rate of 1.0 mL/min to elute others proteins probably bounded onto the column. The eluted volume was collected into 50 tubes, each containing a 2 mL fraction, using a Superfrac collector from GE Healthcare Biosciences (Sweden). The phytase activity containing fractions were pooled.

2.6 Cromatofocusing

The pool of the previous step was loaded onto a Mono PTM 5/50 GL PBE 94 column in 2 mL portions (Amersham Biosciences, Sweden). The separation was performed using a FPLC system, a flow rate of 1 mL/min with a pH range from 6.2 to 4. The column was equilibrated with start buffer (25mM imidazole-HCl pH=6.2). The sample pH was adjusted with start buffer until 6.2. Elution buffer (3 mL) was applied to get a pre-gradient volume before pH gradient begins. A sample with 2 mL was loaded into the column. The elution buffer (polybuffer-74 pH=4.0 HCl 1:8 - Amersham Biosciences, Sweden) was applied during 30 min at flowrate of 1 mL/min. The column was washed with 2 bed-volumes of 2M NaCl to elute any material still bounded onto the column. The procedure of this specific chromatography

consisted on decrease the pH from 6.2 to 4.0. Phytase-active fractions were collected, pooled and lyophilized.

2.7 SDS-PAGE eletrophoresis

SDS-PAGE (sodium dodecyl sulphate polyacrylamide gel electrophoresis) was performed on a gel of 0.1 mm thick and it was carried out using 10% (w/v) separating gelat 150V according to Laemmli method (Laemmli 1970). Gels were stained with Coomassie brilliant blue R-250. Molecular markers used awere SDS6H2 (Sigma) containing six proteins (myosin, α -galactosidase, phosphorylase b, bovine albumin, ovalbumin, carbonic anhydrase).

2.8 Substrate affinity

Phytase activity was measured using method described above except that phytate was replaced with different phosphorylated compounds. To test the pure enzyme-substrate affinity and substrate selectivity, five compounds were selected, Na-phytate, the original substrate, 4-nitrophenyl phosphate, 1-naphtyl phosphate, adenosine 5-triphosphate (ATP) and adenosine 5-diphosphate (ADP) at concentrations 0.5, 0.5, 0.8, 1.0, 1.5, 2.0, 3.0, 4.0 and 5.0 mM. All substrates were purchased from Sigma.

2.9 Optimum pH

Optimum pH of pure phytase activity was determined using different buffers and the standard assay: pH 1.0-3.5 (0.1M glycine-HCl), pH 3.5-6.0 (0.1M sodium acetate-HCl), pH 6.0-7.0 (0.1M Tris-acetic acid), pH 7.0-9.0 (0.1M Tris-HCl), pH 9.0-10.0 (0.1 M glycine-NaOH). Phytase assays were performed from pH 1.0 to 10.0.

2.10 Optimum temperature

Six different temperatures were tested to define the optimal temperature for enzymatic phytate dephosphorylation. The temperatures used were 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75 and 80°C. The tests were performed in standard buffer.

2.11 Km for substrates

In order to evaluate the substrate affinity and substrate selectivity, five compounds were selected: the original substrate, Na-phytate, naphtyl-1 phosphate, *p*-nitrophenylphosphate,

ATP and ADP. Enzyme activity was determined using a serial dilution of the substrate. The kinetic parameters were determined using Lineweaver-Burk plots.

2.12 Stability of pure phytase at 4°C

After different incubation times at 4°C, crude enzymatic extract containing phytase produced by *Aspergillus niger* FS3, lost 41% of its activity after 72 hours when kept in pH 2.85, and 52% when at pH 7.0.

2.13 Phytase thermal stability

Phytase thermostability was estimated at different temperatures (60, 70 and 80°C) using the standard buffer. A diluted sample (1 ml) was added into 2mL eppendorf tubes pre-incubated at 60, 70, and 80°C. The process was stopped transferring the tubes into ice bath immediately during 5 min, performing 40 minutes of total time of thermal treatment. The residual phytase activity was estimated with phytase assay protocol.

All experiments were done in triplicate and the average and standard deviation values were reported.

3. Results and Discussion

3.1 Phytase purification

The phytase was purified using cation, anion-exchange chromatography and chromatofocusing. Cation-exchange chromatography profile shows phytase activities (U/ml), OD₂₈₀ and NaCl gradient (Fig. 1). Under these conditions phytase activity was detected as a single activity peak at the elution volume from 240 to 360 mL (corresponding to fractions from 24 to 36), with total activity of 170.4 U. According Ullah & Gibson (Ullah and Gibson, 1987), phytase from *A. ficcum* was eluted at 100 mM in a cation-exchange chromatography using a smaller column SP-Trisacryl M, while *A. niger* FS3 phytase was eluted in one peak after 275 mM NaCl. OD₂₈₀ data indicates four peaks, but the maximum activity of phytase was detected along the second peak.

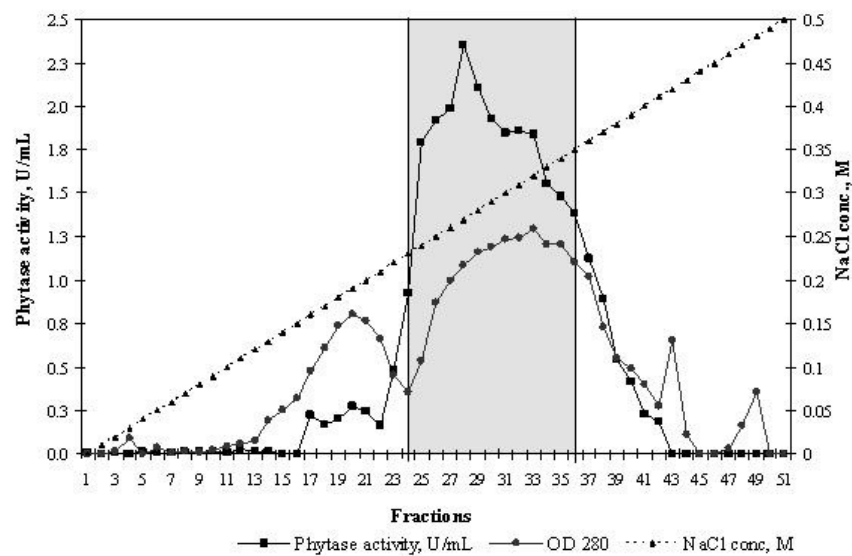


Fig. 1 - SP sepharose FF column. Spot region shows the active fractions.

Fig. 2 shows the elution profile of phytase. A peak of phytase activity was eluted after 150mM and 170mM NaCl, respectively.

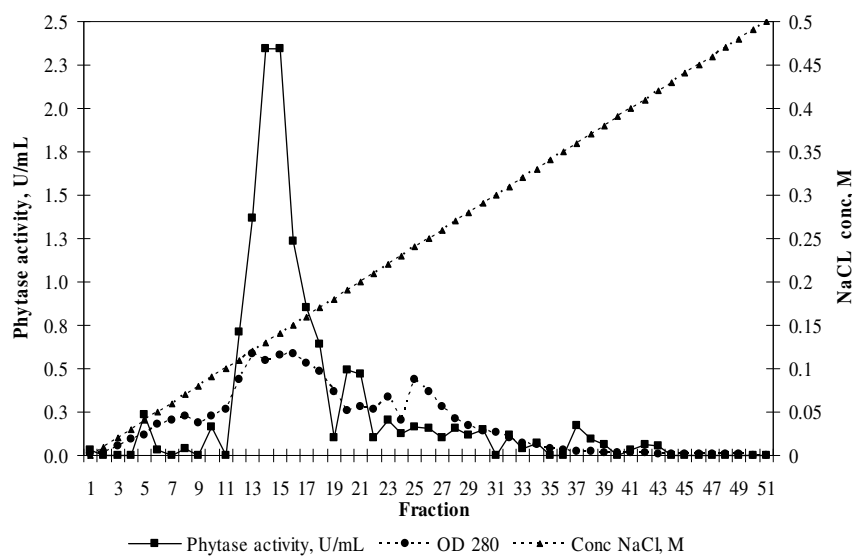


Fig. 2 - Mono Q column profile of phytase elution

Chromatofocusing results (Fig. 3) show the profile of phytase elution. A single peak of high phytase activity was observed in fraction 7 and 8, which elution pH of 5.4-5.6 corresponds to the protein isoelectric point.

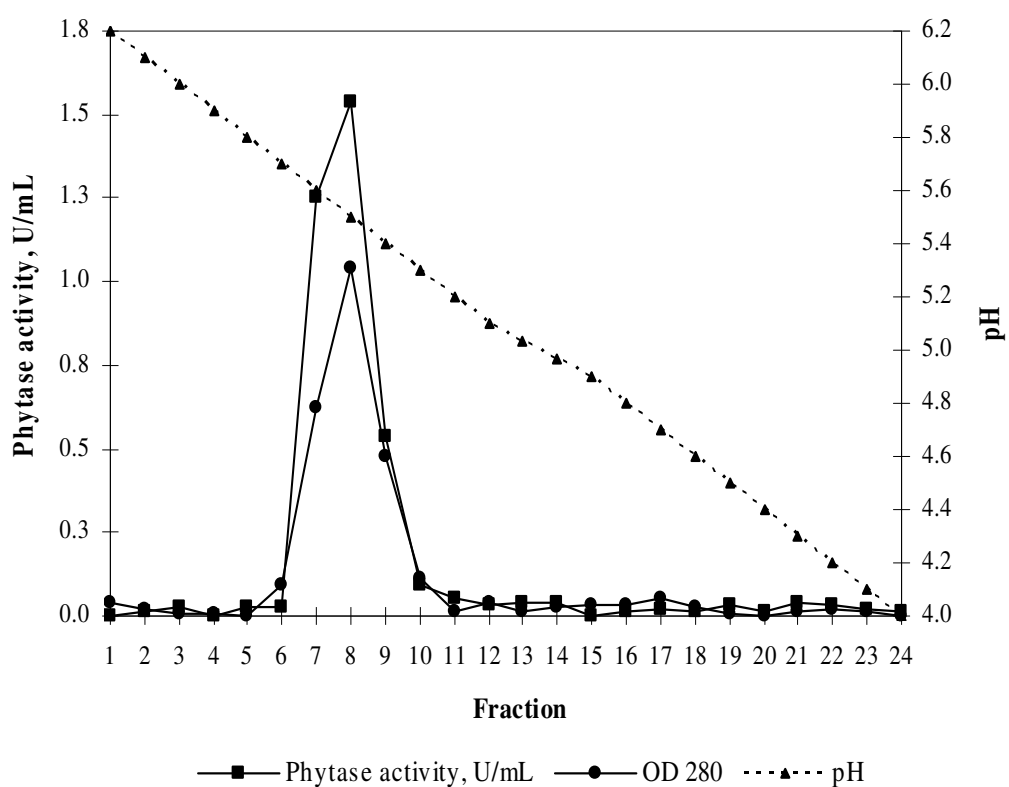


Fig. 3 - Mono P column profile of chromatofocusing

Table 1 - Summary of the purification of *A. niger* FS3 phytase

Step	Volume (mL)	Total protein (mg)	Specific activity (U/mg)	Yield (%)	Purification (fold)
Crude extract	400	28.92	8.57	100	1.0
SP sepharose	120	8.85	19.25	68.71	2.25
Mono Q	23.6	2.41	21.56	20.93	2.51
Mono P	2.5	0.18	86.3	6.35	10.1

The results of phytase purification generated from citric pulp fermentation by *A. niger* FS3 are summarized in Table 1. Phytase was purified 10.1-fold from crude extract supernatant with 6.35% recovery. The purified enzyme showed a specific activity of 86.3 U/mg for sodium phytate hydrolysis. The specific activity of the purified phytase (86.3 U/mg) is higher than the specific activities previously reported for fungal phytases of *Rhizopus oligosporus* (11.82 U/mg) (Casey and Walsh 2004), *A. niger* (50-103 U/mg) (Ullah and Gibson 1987, Wyss et al 1999), *A. fumigatus* (23-28 U/mg) (Ullah and Gibson 1987) which showed a single band on SDS-PAGE.

3.2 SDS-PAGE electrophoresis

SDS-PAGE (Fig. 4) shows the phytase samples obtained at the steps of phytase purification. Compared to crude enzyme solution, which presented a high protein concentration (lane 1 and 2), the lane 3 represents phytase pooled from SP-sepharose cationic chromatography column. Lane 4 represents phytase pooled from Mono Q and Lane 5 from chromatofocusing. SDS-PAGE gel presented 16 and 15 bands in lane 1 and 2, respectively. After the cationic exchange chromatography, the gel has 6 bands, showing cationic exchange chromatography effectiveness to the first step of the purification process. After chromatofocusing the phytase is pure, showing molecular mass around 100 kDa.

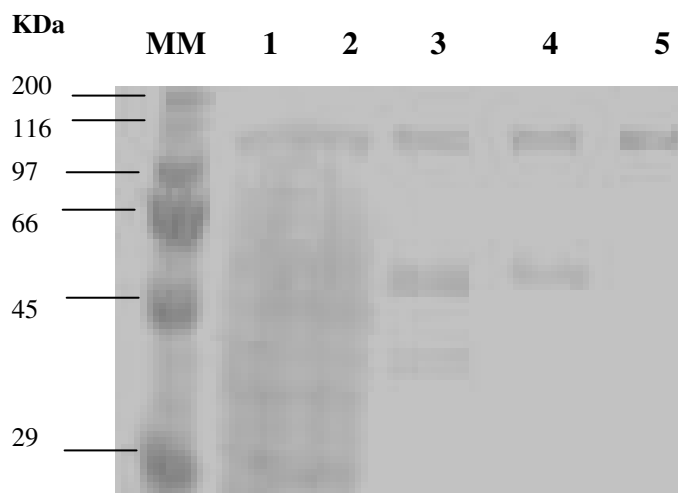


Fig. 4 - SDS-PAGE analysis of the *A. niger* FS3 phytase in the crude extract (lanes 1 and 2), SP-sepharose column (lane 3), Mono Q column (lane 4) and after chromatofocusing Mono Q (lane 5). Molecular weight marker (SDS6H2, Sigma) (lane MM) comprising myosin (200), β -galactosidase (116), phosphorylase b (97), albumin (66), ovalbumin (45) and carbonic anhydrase (29).

Aspergillus niger phytase (EC 3.1.3.8) is an extra cellular glycoprotein, with a mass of around 80 kDa (Ullah and Gibson 1987). Zhang et al (Zhang et al 2005) studied a 68.5 kDa phytase from *A. ficuum* AS3.324. Lassen et al (2001) identified a phytase from *A. niger* with 66.36 kDa. Others phytase were also characterized: *A. niger* phytase with approximately 100kDa (Dvorakova et al 1997), *A. niger* commercial phytase (F. Hoffmannp-La Roche) 89.14 and *A. niger* commercial phytase (BASF, Netherlands) 65.73 kDa (Yin et al 2007), *A. niger* SK-57 60 kDa (Nagashima et al 1999), *A. niger*-307 39.0 kDa (Sariyska et al 2005), *Cladosporium* sp. FP-1 32.6 kDa (Quan et al 2004). Han and Lei proved that glycosylation increases molecular weight of phytase (Han et al 1999).

3.3 Characterization of pure enzyme

3.3.1 Substrate affinity

The effect of different substrates concentration on phytase activity is shown at Fig. 5. In order to find the substrate affinity and substrate selectivity, Na-phytate, naphthyl-1 phosphate, p-nitrophenylphosphate, ATP and ADP were tested. Na-phytate was the substrate with the higher specificity, showing the highest velocity ($3.077 \mu\text{mol}$ of inorganic-P liberation/ml.min) with 3 mM of phytate concentration. Fig 6 and 7 show the results of the experiments presenting both, velocity and Lineweaver-Burk plots, to provide a precision determination of V_{max} and K_m .

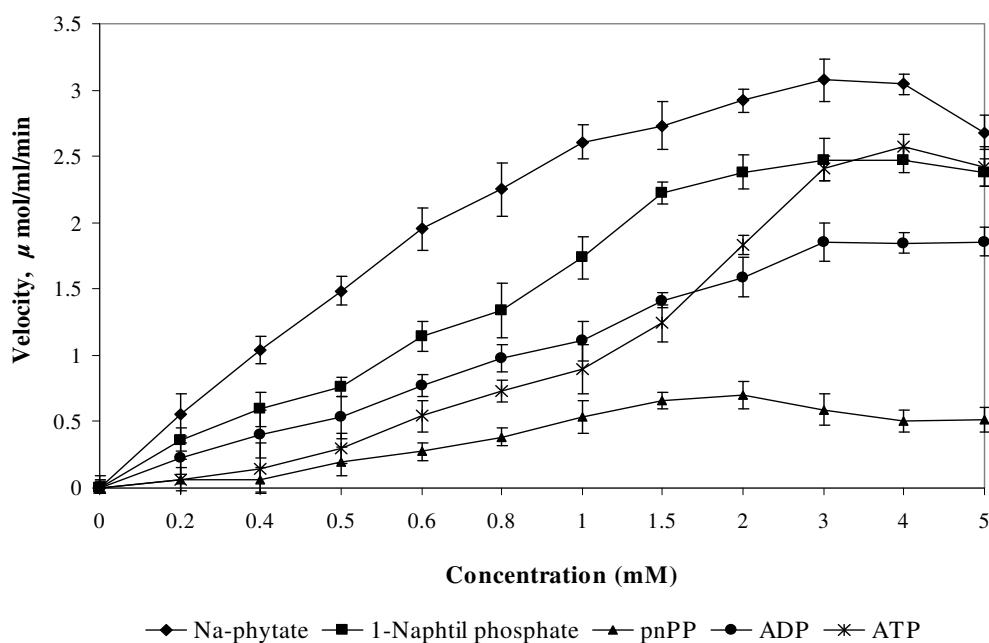


Fig. 5 - Effect of substrate concentration on phytase activity

Plotting the reciprocals of the same data points yields a double-reciprocal (Lineweaver-Burk plot) is presented in Fig.6 to provide a more precise determination of V_{max} and K_m of Na-phytate, 1-Naphtylphosphate, ATP, ADP and pnPP.

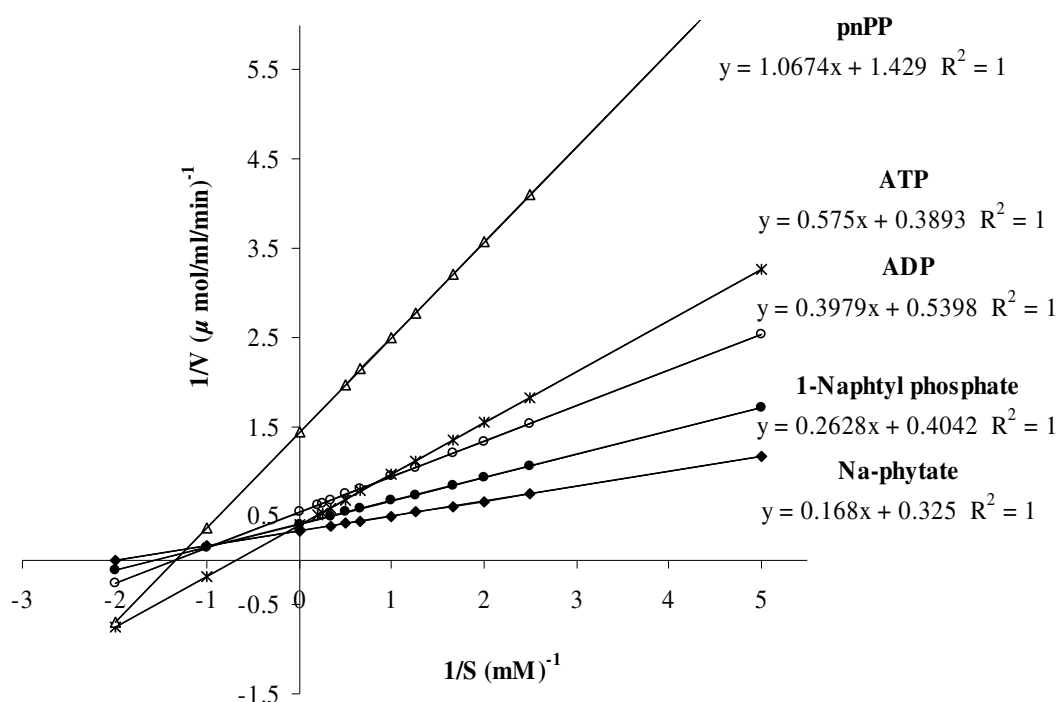


Fig. 6 - Lineweaver-Burk plot

K_m and V_{max} determination: The determination of the substrate selectivity of the phytase phosphorylated substrates were utilised for determinations. Typical Michaelis-Menten kinetics for all compounds tested was achieved for the phytase which has broad substrate specificity. Phytase exhibited the highest specificity constant (V_{max}/K_m) equivalent to 5.9 for Na-phytate compared to the others substrates tested (Table 2), 3.8 for naphtyl 1-phosphate, 1.75 for ATP, 2.5 for ADP, 0.93 for pnPP, showing that Na-phytate might be the *in vivo* substrate of this enzyme.

According to the literature, phytase specificity constants can be different. Phytase of *A. niger* $K_m=0.010-0.040$ mM (Konietzny and Greiner 2002), $K_m=0.44$ of *A. niger*-92 (Dvorakova et al 1997), $K_m=0.04$ mM of *A. ficuum* (Ullah and Gobson 1987), *A. fumigatus* <0.010 mM (Pasamontes et al 1997), *A. terreus* $0.011-0.023$ mM (Wyss et al 1999).

Table 2 - K_m and V_{max} determined through Lineweaver-Burk linearization

Substrate	$1/V_{max}$	V_{max}	$-1/K_m$	K_m	Slope (K_m/V_{max})	V_{max}/K_m
Na-phytate	0.32	3.07	-1.93	0.52	0.169	5.9
Naphthyl 1-phosphate	0.40	2.47	-1.54	0.65	0.26	3.8
ATP	0.39	2.57	-0.68	1.47	0.57	1.75
ADP	0.54	1.85	-1.35	0.74	0.40	2.5
pnPP	1.43	0.70	-1.33	0.75	1.07	0.93

3.3.2 Optimum pH

The best pH for phytase activity was determined using different pH-values for activity assay (Fig. 7). The best phytate dephosphorylation was achieved at pH 5.5. It is in accordance with the optimum pH ranges 5.0–5.5 for the most fungal phytases (Yin et al 2007). Phytase was virtually inactive above pH 8.0 and below pH 2.0. Similar results were already reported by Ullah and Gibson (1987) for the phytase from *A. ficuum*. One of the enzymes produced by *A. ficuum* exhibited optimal pH at pH 5.0-5.5. For the phytase of *A. fumigatus* only a single pH optimum at pH 5.0 was found (Ullah et al 2000).

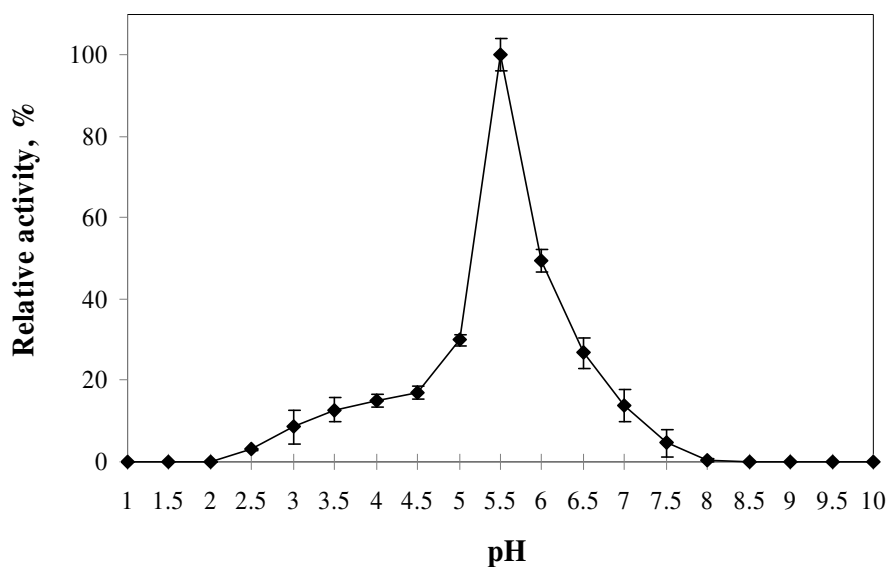


Fig. 7 - Optimum pH of pure phytase

3.3.3 Optimum temperature

Optimal temperature for phytate dephosphorylation was showed to be 60°C (Fig. 8). The activity at 60°C was 15% higher than at 50°C, the temperature used in the standard phytase assay. An abrupt decrease in enzymatic activity was observed above 60°C. At 70°C, approximately 40% of the maximal activity remained.

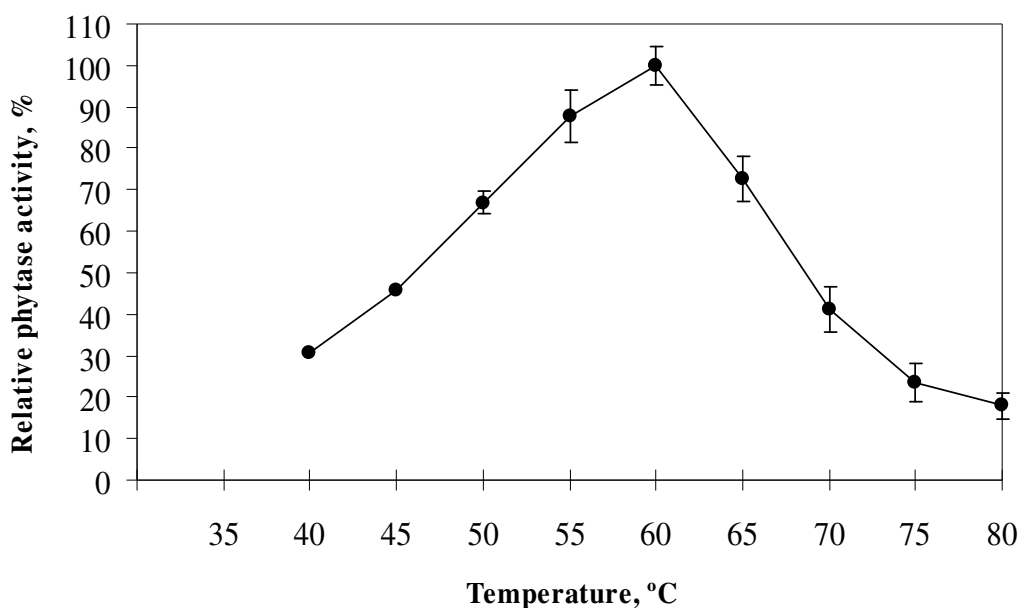


Fig. 8 - Optimum temperature of pure phytase.

Most of the microbial phytases studied, showed similar optimal temperature (Konietzny and Greiner 2002), temperature range of 45 to 55°C (Boyce and Walsh 2007) and thermal inactivation at 70°C (Woodzinski and Ullah 1996). The phytase studied in this work seems to be more resistant to higher temperature than the phytase from *A. ficuum* (Ullah and Gibson 1987). However this enzyme was almost completely inactive at 70°C.

The pH range obtained is in accordance with Konietzny & Greiner (2002), which for most of the phytate-degrading enzymes described so far, belongs to the acidic type, and their optimal pH ranges from 4.5 to 6.0. According to the optimum pH, the produced phytase could be classified as an acid phytase with an optimum pH range between 3.5-6.0 (Greiner and Konietzny 2006).

3.3.4 Stability of pure phytase at 4°C

Crude enzymatic extract containing phytase produced by *Aspergillus* sp. FS3, was stored at 4°C for different times; when kept in pH 2.85, the enzyme lost 41% of its activity after 72 hours and 52% when at pH 7.0 (Fig. 9). It can be concluded that at acid conditions the phytase showed a higher stability compared to basic conditions.

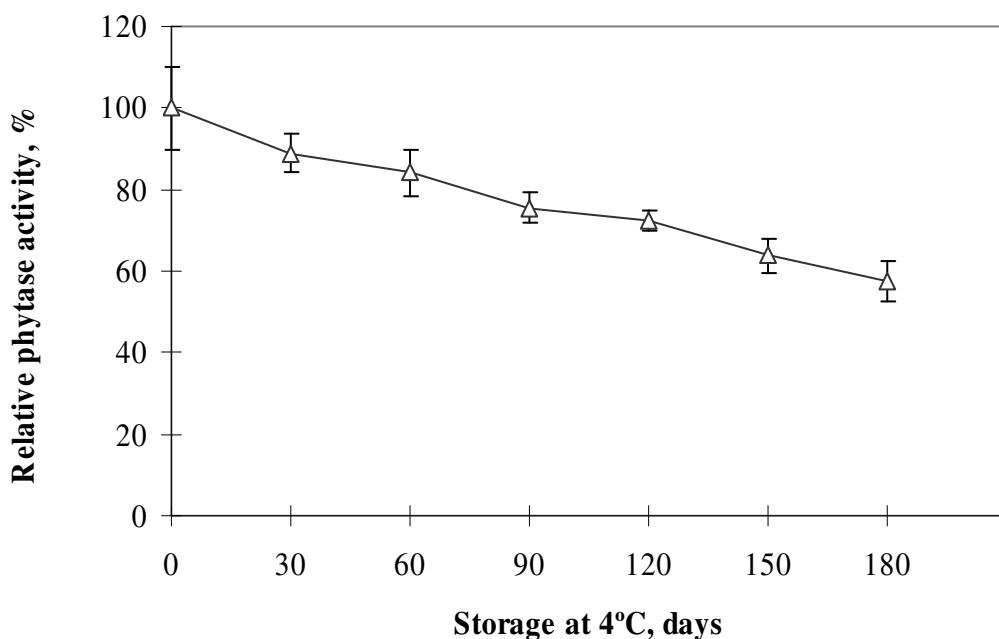


Fig. 9 - Stability of pure phytase at 4°C

3.3.5 Thermal stability of pure phytase at 60, 70 and 80°C

Pure phytase was incubated at 60, 70, and 80°C and showed different stability behavior (Fig.10). Phytase conditioned at 60°C showed to be able to withstand 10, 20, and 40 minutes with an activity residual of 86.0, 68.4 and 48.2%, respectively. Showing lower stability at 80°C, phytase retained 30.5% after 10 minutes. Phytase of *Aspergillus* sp. FS3 is more stable than *A. ficcum* AS3.324 phytase (Zhang et al 2005). Phytase from *E. coli* maintained only 24% activity when exposed to 60°C for 1h and lost completely phytase activity at 70°C. For instance, after 80°C of heat treatment during 15 min, residual enzyme activity was 25.1% of the initial. Under the same condition, the thermal stability of this enzyme was higher than residual phytase activity of *A. ficuum* AS3.324 that presented only 5.5% (Zhang et al 2005),

phytase activity of *E. coli*, 15% (Luo et al 2004); phytase of *A. niger* retained 52% of its activity at 80°C for 15 min (Bei et al 2001, Igbasan et al 2000).

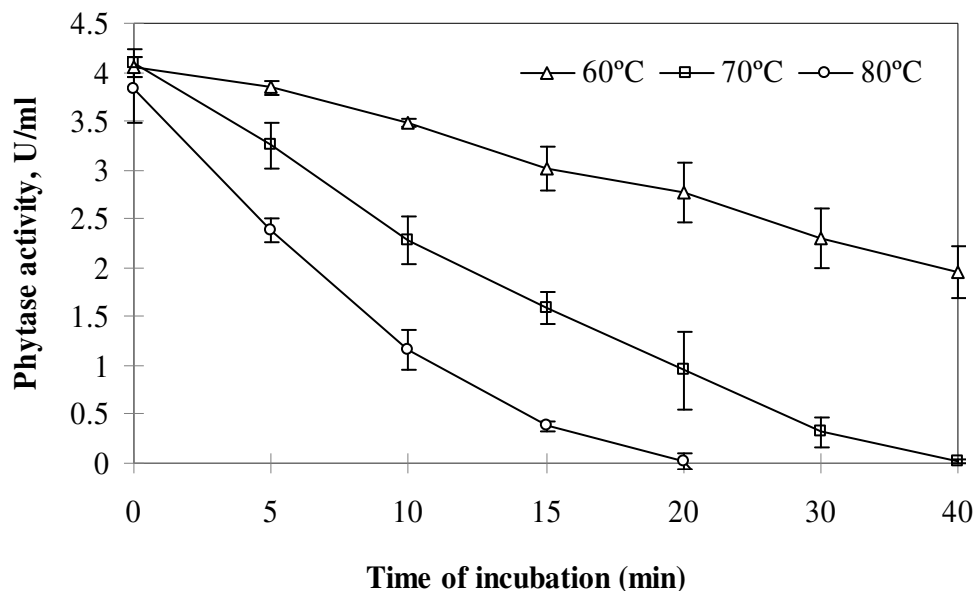


Fig. 10 - Thermal stability of pure phytase at 60, 70, and 80°C

4. Conclusion

An enzyme which has commercial interest should fulfill a plenty of predefined quality criteria. The studies on pure phytase from *A. niger* FS3 indicated that citric pulp may be an cheap alternative substrate to synthesize phytase once it exhibits suitable properties for biotechnological application for instance in food processing. It was shown that the enzymatic properties are similar to the commercialized phytase from *A. ficuum*. In addition, the purified phytase in this work is sufficiently able to withstand pelleting temperatures, exhibiting sufficiently high phytate-degrading activity.

References

IBGE, Brazilian Institute of Geography and Statistic. [On line] <<http://www.ibge.gov.br>> (2007).

Abecitrus, The Brazilian Association of Citrus Exporters. [On line] <<http://www.abecitrus.com.br>> (2008).

IBGE (2008). Brazilian Institute of Geography and Statistic. Agroindustry production. [On line] <<http://www.ibge.gov.br>> (2007).

Nelson PE, Tressler DK (1980), Fruit and Vegetable Juice Processing Technol., AVI Publishing Co., Westport, USA, 603p.

Spier MR, Greiner R, Rodríguez-León JA, Woiciechowski AL, Pandey A, Soccol VT, Soccol CR (2008), Phytase production using citric pulp and other residues of the agro-industry in SSF by fungal isolates, Food Technol. Biotechnol. 46, 176-180.

Citroex, Concentrates and Essential Oils Company, Brazil (2008).

Tang S, Boehme L, Lam H, Zhang Z, *Pichia pastoris* fermentation for phytase production using crude glycerol from biodiesel production as the sole carbon source, Biochem. Eng. J. doi:10.1016/j.bej.2008.09.020.

Singh B, Satyanarayana T (2008), Phytase production by a thermophilic mould *Sporotrichum thermophile* in solid state fermentation and its potential applications, Bioresource Technol. 99, 2824–2830.

Roopesh K, Ramachandran S, Nampoothiri KM, Szakacs G, Pandey A (2006), Comparison of phytase production on wheat bran and oilcakes in solid-state fermentation by *Mucor racemosus*, Bioresource Technol. 97, 506–511.

Fujita J, Shigeta S, Yamane Y, Fukuda H, Kizaki Y, Wakabayashi S, Ono K (2003), Production of two types of phytase from *Aspergillus oryzae* during industrial koji making. J. Biosci. Bioeng. 95, 460-465.

Casey A, Walsh G (2003), Purification and characterization of extracellular phytase from *Aspergillus niger* ATCC 9142, Bioresource Technol. 86, 183–188.

Quan CS, Tian WJ, Dan SD, Kikuchi JI (2004), Purification and Properties of a Low-Molecular-Weight Phytase from *Cladosporium* sp. FP-1, J. Biosci. Bioeng. 97, 260–266.

Li XY, Liu Q, Chi ZM (2008), Production of phytase by a marine yeast *Kodamaea ohmeri* BG3 in an oats medium: Optimization by response surface methodology, Bioresource Technol. 99, 6386–6390.

Vats P, Banerjee UC (2002), Studies on the production of phytase by a newly isolated strain of *Aspergillus niger* var teigham obtained from rotten wood-logs, Process Biochem. 38, 211-217.

Quan CS, Zhang LH, Wang YJ, Ohta Y (2001), Production of phytase in a low phosphate medium by a novel yeast *Candida krusei*, J. Biosci. Bioeng. 92, 154–60.

Bradford M (1976), A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding, Anal. Biochem. 72, 248-258.

Heinonen JK, Lahti RJ (1981), A new and convenient colorimetric determination of inorganic orthophosphate and its application to the assay of inorganic pyrophosphatase, Analytical Biochem. 113, 313-317.

Laemmli UK (1970), Cleavage of structural proteins during the assembly of the head of bacteriophage T4, Nature 227, 680-5.

Ullah AHJ, Gibson DM (1987), Extracellular phytase (E. C. 3. 1. 3. 8) from *Aspergillus ficuum* NRRL 3135: purification and characterization, Prep. Biochem. 17, 63–91.

Casey A, Walsh G (2004), Identification and characterization of a phytase of potential commercial interest, *J. Biotechnol.* 110, 313-322.

Wyss M, Brugger R, Kronenberger A, Remy R, Fimbel R, Oesterheld G, Lehmann M, Loon APGM (1999), Biochemical characterization of fungal phytases (Myo-inositol hexakisphosphate phosphohydrolases): catalytic properties, *Appl. and Environ. Microbiol.* 65, 367–373.

Zhang L, Lijia A, Xiaorong G, Yunji W (2005), Properties of *A. ficuum* AS3.324 phytase expressed in tobacco, *Process Biochemistry* 40, 213-216.

Lassen SF, Breinholt J, OstergaardPR, Brugger R, Bischoff A, Wyss M, Fuglsang CC (2001), Expression, gene cloning, and characterisation of five novel phytases from four basidiomycete fungi: *Peniophora lycii*, *Agrocybe pediades*, a *Ceripori* sp., and *Trametes pubescens*, *Applied and Environmental Microbiology* 67, 4701–4707.

Dvořáková J, Volfová O, Kopecký J (1997), Characterization of phytase produced by *Aspergillus niger*. *Folia Microbiologica* 42, 349-352.

Yin QQ, Zheng QH, Kang XT (2007), Biochemical characteristics of phytases from fungi and the transformed microorganism, *Animal Feed Sci. and Technol.* 132, 341–350.

Nagashima T, Tange T, Anazawa H (1999), Dephosphorylation of Phytate by Using the *Aspergillus niger* Phytase with a High Affinity for Phytate, *Appl. Environ. Microbiol.* 65, 4682–4684.

Sariyska MV, Gargova SA, Koleva LA, Angelov AI (2005), *Aspergillus niger* phytase: purification and characterization, *Biotechnol. Biotechnol.*, Eq. 19/2005/3.

Quan CS, Tian WJ, Fan SD, Kikuchi JI (2004), Purification and Properties of a Low-Molecular-Weight Phytase from *Cladosporium* sp. FP-1, *J. Biosc. Bioeng.* 97, 260–266.

Han Y, Wilson DB, Lei XG (1999), Expression of an *Aspergillus niger* Phytase Gene (*phyA*) in *Saccharomyces cerevisiae*, *Appl. Environ. Microbiol.* 65, 1915-1918.

Konietzny U, Greiner R (2002), Molecular and catalytic properties of phytate-degrading enzymes (phytases), *Int. J. Food Sci. Technol.* 37, 791–812.

Dvořáková J, Volfová O, Kopecký J (1997), Characterization of phytase produced by *Aspergillus niger*, *Folia Microbiol.* 42, 349-352.

Pasamontes L, Haiker M, Henriquez-Huecas M, Mitchell DB, Loon APGM (1997), Cloning of the phytases from *Emericella nidulans* and the thermophilic fungus *Talaromyces thermophilus*, *Biochimica Biophys. Acta (BBA) - Gene Structure and Expression*, 1353, 217-223

Ullah AHJ, Sethumadhavan K, Lei XG, Mullaney EJ (2000), Biochemical Characterization of Cloned *Aspergillus fumigatus* Phytase (phyA), *Biochem. Biophys. Res. Communications* 275, 279–285.

Boyce A, Walsh G (2007), Purification and characterisation of an acid phosphatase with phytase activity from *Mucor hiemalis* Wehmer, *J. Biotechnol.* 132, 82–87.

Woodzinski RJ, Ullah AHJ (1996), Biochemical Characterization of Cloned *Aspergillus fumigatus* Phytase (phyA), *Adv. Appl. Microbiol.* 42, 263–302.

Greiner R, Konietzny U (2006), Phytase for Food Application, *Food Technol. Biotechnol.* 44, 125–140.

Zhang L, An J, Gao X, Wang Y (2005), Properties of *A. ficuum* AS3.324 phytase expressed in tobacco, *Process Biochem.* 40, 213-216.

Luo HY, Yao B, Yuan TZ, Wang YR, Shi XY, Wu NF, Fan YL (2004), Overexpression of *Escherichia coli* phytase with high specific activity, *Chin. J. Biotechnol.* 20, 78–84.

Bei JL, Chen Z, Yang L, Liao L, Wang XZ, Jiang ZY (2001), Overexpression of artificial synthetic gene of *Aspergillus niger* NRRL3135 phytase in *Pichia pastoris*, *Chin. J. Biotechnol.* 17, 254–258.

Igbasan FA, Manner K, Miksch G, Borriss R, Farouk A, Simon O (2000), Comparative studies on the in vitro properties of phytases from various microbial origins, Arch. Tierernahr. 53, 353–373.

CONCLUSIONS

- The soil isolate *Aspergillus niger* FS3 was identified as the best phytase producer;
- Among substrates tested, citric pulp resulted in higher phytase formation compared to apple bran, soya bran, wheat bran and rice bran. It was also demonstrated that autoclaved release phosphorus from phytate decreasing phytase yield in fermentation process.
- Optimization of phytase production resulted in overall 4.3-fold increase of phytase production proving the utility of statistical tools. Besides, magnesium and zinc showed importance in phytase production due its contribution in increase of 12% in enzyme yield after optimization studies.
- Phytase synthesis is encouraged with forced air in column type bioreactor. This work also showed a simple alternative for biomass determination in citric pulp fermentation than ergosterol extraction and chromatography analysis. The program FERSOL2 estimated process parameters as yield of biomass from oxygen (YX/O), maintenance coefficient (m) and specific growth rate (μ) showing a good correlation between the data and the model.
- Batch studies demonstrated an alternative to test different parameters to perform cationic chromatography quickly before testing in the column. The strong cationic exchanger induced adsorption improvement when parameters stabilised SP-sepharose FF, glycine-HCl pH 2.85, and enzyme dilution of 1:3 (v/v) were applied. The study in tubes achieved 62.07% of phytase bonded and 59.61% of initial phytase activity was recovery by 1M NaCl elution step. This batch study is an easy and fast way to define the previously parameters to run ionic chromatography.
- The studies on pure phytase from *A. niger* FS3 indicated that citric pulp may be an alternative as substrate to synthesize phytase once it exhibits suitable properties for biotechnological application for instance in food processing. It was shown that the enzymatic properties are similar to the commercialized phytase from *A. ficuum*. In addition, the purified phytase in this work is sufficiently able to withstand pelleting temperatures, exhibiting sufficiently high phytate-degrading activity.

SUGESTIONS TO FUTURE WORK

- Isolate and select new microorganisms still not reported in literature for finding a new phytase which presents higher thermal tolerance and stability during shelf-life;
- Engineering of microorganisms phytases producers in order to optimise their catalytic features as a promising strategy to enhance of thermal tolerance and increase in specific activity - two important issues for animal feed and for food processing applications of phytases;
- Improve pure phytase stability applying stabilizers and drying processes as well as spray-drying and lyofilization.
- Study phytase production in submerged fermentation and performe respirometry analysis.
- Study and characterize the products of IP6 degradation by *A. niger* FS3 phytase.

REFERENCES

- Reddy NR, Sathe SK, Salunkhe DK (1982), Phytate in legumes and cereals. *Advances in Food Research* 28, 1-92.
- Shamsuddin AM (2002), Anti-cancer function of phytic acid. *Int J Food Sci Technol.* 37, 769p.
- Dost K, Tokul O (2006), Determination of phytic acid in wheat and wheat products by reverse phase high performance liquid chromatography. *Analytica Chimica Acta* 558, 22-27.
- Kerovuo J, Rouvinen J, Hatzack F (2000), Analysis of myo-inositol hexakisphosphate hydrolysis by *Bacillus phytase*: indication of a novel reaction mechanism. *Biochemistry Journal*, 352, p. 623-628.
- Loewus F (2002), Biosynthesis of Phytate in Food Grains and Seeds. In: *Food Phytates*, N.R. Reddy, S.K. Sathe (Eds.), CRC Press, Boca Raton, Florida, USA, 53–61.
- Reddy NR (2002), Occurrence, Distribution, Content, and Dietary Intake of Phytate. In: *Food Phytates*, N.R. Reddy, S.K. Sathe (Eds.), CRC Press, Boca Raton, Florida, USA, 25–51.
- Greiner R, Konietzny U (2006), Phytase for Food Application, *Food Technol. Biotechnol.* 44 (2), 125–140.
- Rickard ES, Thompson LU (1997), Interactions and effects of phytic acid. In: Shahidi (Ed), *Antinutrients and phytochemicals in food* (294-313). Washington DC: American Chemical Society.
- Cheryan M, Phytic acid interactions in food systems, *Crit. Rev. Food Sci. Nutr.* 13 (1980) 297–355.

Konietzny U, Greiner R (2003), Phytic Acid: Nutritional Impact. In: Encyclopedia of Food Science and Nutrition, B. Caballero, L. Trugo, P. Finglas (Eds.), Elsevier, London, UK (2003), 4555–4563.

Lopez HW, Leenhardt F, Coudray C, Rémésy C (2002), Minerals and phytic acid interactions: Is it a real problem for human nutrition?, *Int. J. Food Sci. Technol.* 37, 727–739.

Wang M, Hettiarachchy NS, Qi M, Burks W, Siebenmorgen T (1999), Preparation and functional properties of rice bran protein isolate, *J. Agric. Food Chem.* 47 (1999), 411–416.

Fredrikson M, Biot P, Alminger ML, Carlsson NG, Sandberg AS (2001), Production process for high-quality pea-protein isolate with low content of oligosaccharides and phytate, *J. Agric. Food Chem.* 49, 1208–1212.

Caransa A, Simell M, Lehmussaari M, Vaara M, Vaara T (1988), A novel enzyme application in corn wet milling, *Starch*, 40, 409–411.

Antrim RL, Mitchinson C, Solheim LP (1997), Method for liquefying starch. US patent 5652127.

Kvist S, Carlsson T, Lawther JM, DeCastro FB (2005), Process for the fractionation of cereal brans. US patent application US 20050089602 (2005).

Chen QC, Lib BW (2003), Separation of phytic acid and other related inositol phosphates by high-performance ion chromatography and its applications. *Journal of Chromatography A* 1018(1), 41-52.

Greiner R (2007), Inositol Phosphates: Linking Agriculture and the Environment. Phytate-degrading enzymes: regulation of synthesis in microorganisms and plants (3), 78-95.

Mullaney EJ, Ullah AHJ (2007), Inositol Phosphates: Linking Agriculture and the Environment. Phytases: Attributes, catalytic mechanisms and applications.

Barrientos L, Scott JJ, Murthy PP (1994), Specificity of hydrolysis of phytic acid by alkaline phytase from lily pollen. *Plant Physiology* 106, 1489-1495.

Greiner R, Carlsson NG, Alminger ML (2000), Stereospecificity of myo-inositol hexakisphosphate dephosphorylation by a phytate-degrading enzyme of *Escherichia coli*. *Journal of Biotechnology* 84, 53-62.

Konietzny U, Greiner R (2002), Molecular and catalytic properties of phytate-degrading enzymes (phytases). *Int J Food Sci Technol* 37, 791-812.

Andlid T, Veide J, Sandberg AS (2004), Metabolism of extracellular inositol hexaphosphate (phytate) by *Saccharomyces cerevisiae*. *International Journal of Food Microbiology* 97, 157–169.

Kim DS, Godber JS, Kim HR (1999), Culture conditions for a new phytase-producing fungus. *Biotechnology Letters* 21, 1077-1081.

Sano K, Fukuhara H, Nakamura Y (1999), Phytase of the yeast *Arxula adeninivorans*. *Biotechnology Letters* 21, 33-38.

Shieh TR, Ware JH (1968), Survey of microorganisms for the production of extracellular phytase, *Appl Environ Microbiol.* 1968 September; 16(9), 1348-1351.
Sano K, Fukuhara H, Nakamura Y (1999), Phytase of the yeast *Arxula adeninivorans* *Biotechnol Letters* 21, 33–38.

Vats P, Banerjee UC (2002), Studies on the production of phytase by a newly isolated strain of *Aspergillus niger* var *teigham* obtained from rotten wood-logs, *Process Biochem.* 38, 211-217.

Han YW, Gallagher DJ (1987), Phosphatase production by *Aspergillus ficuum*. *J Industrial Microbiol* 1, 295-301.

Lambrechts C, Boze H, Segueilha L, Moulin G, Galzy P (1993), Influence of culture conditions on the biosynthesis of *Schwanniomyces castellii* phytase. *Biotechnol Letters* 15, 399-404.

Nayini NR, Markakis P (1984) The phytase of yeast. *Lebensm Wiss Technol* 17, 24-26.

Yamada K, Minoda Y, Kobayashi T, Hidaka Y, Matuo H, Kobayashi M (1968), Phytase from *Aspergillus terreus* I production, purification and some general properties of the enzyme. *Agric Biol Chem* 32, 1275-1282.

Mullaney EJ, Daly CB, Ullah AH (2000), Advances in phytase research. *Adv Appl Microbiol*, 47, 157-99.

Sandberg AS, Andlid T (2002), Phytogenic and microbial phytases in human nutrition. *Int J Food Sci Technol* 37, 823-833.

Wise A, Gilbert DJ (1982), Phytate hydrolysis by germ-free and conventional rats. *Appl Environ Microbiol* 43, 753-756.

Rodriguez E, Wood ZA, Karplus PA, Lei XG (2000), Site-directed mutagenesis improves catalytic efficiency and thermostability of *Escherichia coli* pH 2.5 acid phosphatase/phytase expressed in *Pichia pastoris*, *Arch. Biochem. Biophys.* 382, 105-112.

Garrett JB, Kretz KA, O'Donoghue E, Kerovuo J, Kim W, Barton NR, Hazlewood GP, Short JM, Robertson DE, Gray KA (2004), Enhancing the thermal tolerance and gastric performance of a microbial phytase for use as a phosphate-mobilizing monogastric-feed supplement, *Appl. Environ. Microbiol.* 70, 3041-3046.

Shears SB (1998), The versatility of inositol phosphates as cellular signals, *Biochim. Biophys. Acta*, 1436, 49–67.

Konietzny U, Greiner R (2003), Phytic Acid: Nutritional Impact. In: *Encyclopedia of Food Science and Nutrition*, B. Caballero, L. Trugo, P. Finglas (Eds.), Elsevier, London, UK, 4555–4563.

Lopez HW, Leenhardt F, Coudray C, Rémésy C (2002), Minerals and phytic acid interactions: Is it a real problem for human nutrition?, *Int. J. Food Sci. Technol.* 37, 727– 739.

Sandberg AS, Brune M, Carlsson NG, Hallberg L, Skoglund E, Rossander-Hulthén L (1999), Inositol phosphates with different numbers of phosphate groups influence iron absorption in humans, *Am. J. Clin. Nutr.* 70, 240–246.

Sandström B, Sandberg AS (1992), Inhibitory effects of isolated inositol phosphates on zinc absorption in humans, *J. Trace Elem. Electrol. Health Dis.* 6, 99–103.

Han O, Failla ML, Hill AD, Morris ER, Smith Jr JC (1994), Inositol phosphates inhibit uptake and transport of iron and zinc by a human intestinal cell line, *J. Nutr.* 124, 580–587.

Haros M, Rosell CM, Benedito C (2001), Fungal phytase as a potential breadmaking additive, *Eur. Food Res. Technol.* 213, 317–322.

Wang M, Hettiarachchy NS, Qi M, Burks W, Siebenmorgen T (1999), Preparation and functional properties of rice bran protein isolate, *J. Agric. Food Chem.* 47, 411–416.

Caransa A, Simell M, Lehmussaari M, Vaara M, Vaara T (1988), A novel enzyme application in corn wet milling, *Starch*, 40, 409–411.

Antrim RL, Mitchinson C, Solheim LP (1997), Method for liquefying starch. US patent 5652127.

Kvist S, Carlsson, Lawther JM, DeCastro FB (2005), Process for the fractionation of cereal brans. US patent application US 20050089602.

Loewus F (2002), Biosynthesis of Phytate in Food Grains and Seeds. In: *Food Phytates*, N.R. Reddy, S.K. Sathe (Eds.), CRC Press, Boca Raton, Florida, USA, 53–61.

Maffucci T, Piccolo E, Cumashi A, Iezzi M, Riley AM, Saiardi A, Godage HY, Rossi C, Brogгинi M, Iacobelli S, Potter BVL, Innocenti P, Falasca M (2005), Inhibition of the

phosphatidylinositol-3-kinase/Akt pathway by inositol pentakisphosphate results in antiangiogenic and antitumor effects, *Cancer Res.* 65,8339–8349.

Carrington AL, Calcutt NA, Ettlinger CB, Gustafsson T, Tomlinson DR (1993), Effects of treatment with myo-inositol or its 1,2,6-trisphosphate (PP56) on nerve conduction in streptozotocin-diabetes, *Eur. J. Pharmacol.* 237, 257–263.

Claxon AC, Morris, D. Blake, M. Siren, B. Halliwell, T. Gustafsson, B. Löfkvist, I. Bergelin (1990), The anti-inflammatory effects of D-myo-inositol-1,2,6-trisphosphate (PP56) on animal models of inflammation, *Agents Actions*, 29, 68–70.

Grases F, J.G. March, R.M. Prieto, B.M. Simonet, A. Costa-Bauza, A. García-Raja, Conte A (2000), Urinary phytate in calcium oxalate stones formers and healthy people, *Scand. J. Urol. Nephrol.* 34, 162–164.

Jariwalla RJ, R. Sabin, S. Lawson, Z.S. Herman, Lowering of serum cholesterol and triglycerides and modulation of divalent cations by dietary phytate, *J. Appl. Nutr.* 42 (1990) 18–28.

Vucenik I, Shamsuddin AM (2003), Cancer inhibition by inositol hexaphosphate (IP6) and inositol: From laboratory to clinic, *J. Nutr.* 133 (Suppl.), 3778–3784.

Lehmann M, Loch C, Middendorf A, Studer D, Lassen SF, Pasamontes L, van Loon APGM, Wyss M (2002), The consensus concept for thermostability engineering of proteins: Further proof of concept, *Protein Eng.* 15, 403–411.

Tomschy A, M. Tessier, M. Wyss, R. Brugger, C. Broger, L. Schnoebele, A.P.G.M. van Loon, L. Pasamontes, (2000a), Optimization of the catalytic properties of *Aspergillus fumigatus* phytase based on the three-dimensional structure, *Protein Sci.* 9 (2000a), 1304–1311.

Tomschy A, M. Wyss, D. Kostrewa, K. Vogel, M. Tessier, S. Höfer, H. Bürgin, A. Kronenberger, R. Rémy, A.P.G.M. van Loon, L. Pasamontes (2000b), Active site residue 297

of *Aspergillus niger* phytase critically affects the catalytic properties, FEBS Lett. 472, 169-172.

Miksch G, S. Kleist, K. Friehs, E. Flaschel (2002), Overexpression of the phytase from *Escherichia coli* and its extracellular production in bioreactors, Appl. Microbiol. Biotechnol. 59, 685-694.

Mayer AF, K. Hellmuth, H. Schlieker, R. Lopez-Ulibarri, S. Oertel, U. Dahlems, A.W.M. Strasser, A.P.G.M. van Loon (1999), An expression system matures: A highly efficient and cost-effective process for phytase production by recombinant strains of *Hansenula polymorpha*, Biotechnol. Bioeng. 63, 373-381.

Yao B, C. Thang, J. Wang, Y. Fan (1998), Recombinant *Pichia pastoris* overexpressing bioactive phytase, Science in China 41, 330-336.

Verwoerd TC, P.A. van Paridon, J.J. van Ooyen, J.W.M. van Lent, A. Hoekema, J. Pen (1995), Stable accumulation of *Aspergillus niger* phytase in transgenic tobacco leaves, Plant Physiol. 109, 1199-1205.

Ullah AHJ, K. Sethumadhavan, E.J. Mullaney, T. Ziegelhoffer, S. Austin-Phillips (1999), Characterization of recombinant fungal phytase (phyA) expressed in tobacco leaves, Biochem. Biophys. Res. Commun. 264, 201-206.

J. Pen, T.C. Verwoerd, P.A. van Paridon, R.F. Beudeker, P.J.M. van den Elzen, K. Geeres, J. van der Klis, H.A.J. Versteegh, A.J.J. van Ooyen, A. Hoekema, Phytase-containing transgenic seeds as a novel feed additive for improvement phosphorus utilization, Bio/Technol. 11 (1993) 811-814.

George TS, R.J. Simpson, P.A. Hadobas, A.E. Richardson (2005), Expression of a fungal gene in *Nicotiana tabacum* improves phosphorus nutrition of plants grown in amended soils, Plant Biotechnol. J. 3, 129-140.

Lung CS, W.L. Chan, W. Yip, L. Wang, E.C. Yeung, B.L. Lim (2005), Secretion of beta-propeller phytase from tobacco and Arabidopsis roots enhances phosphorus utilization, *Plant Sci.* 169, 341-349.

Yip W, L. Wang, C. Cheng, W. Wu, S. Lung, B.L. Lim (2003), The introduction of a phytase gene from *Bacillus subtilis* improved the growth performance of transgenic tobacco, *Biochem. Biophys. Res. Commun.* 310, 1148-1154.

Ullah AHJ, K. Sethumadhavan, E.J. Mullaney, T. Ziegelhoffer, S. Austin-Phillips (2002), Cloned and expressed fungal phyA gene in alfalfa produces a stable phytase, *Biochem. Biophys. Res. Commun.* 290, 1343-1348.

Coello P, J.P. Maughan, A. Mendoza, R. Philip, D.W. Bollinger, T.L. Veum, L.O. Vodkin, J.C. Polacco (2001), Generation of low phytic acid Arabidopsis seeds expressing an *E. coli* phytase during embryo development, *Seed Sci. Res.* 11, 285-291.

Richardson AE, P.A. Hadobas, J.E. Hayes (2001), Extracellular secretion of *Aspergillus* phytase from Arabidopsis roots enables plants to obtain phosphorus from phytate, *Plant J.* 25, 641-649.

Mudge SR, F.W. Smith, A.E. Richardson (2003), Root-specific and phosphate-regulated expression of phytase under the control of a phosphate transporter promoter enables Arabidopsis to grow on phytate as a sole P source, *Plant Sci.* 165, 871-878.

George TS, A.E. Richardson, P.A. Hadobas, R.J. Simpson (2004) Characterization of transgenic *Trifolium subterraneum* L. which expresses phyA and releases extracellular phytase: Growth and P nutrition in laboratory media and soil, *Plant, Cell Environ.* 27, 1351-1361.

Jin UH, J.A. Chun, J.W. Lee, Y.B. Yi, S.W. Lee, C.H. Chung (2004), Expression and characterization of extracellular fungal phytase transformed sesame hairy root cultures, *Protein Expr. Purif.* 37, 486-492.

Li J, C.E. Hegemann, R.W. Hanlon, G.H. Lacy, D.M. Denbow, E.A. Grabau (1997), Secretion of active recombinant phytase from soybean cell-suspension cultures, *Plant Physiol.* 114, 1103-1111.

Ponstein AS, J.B. Bade, T.C. Verwoerd, L. Molendijk, J. Storms, R.F. Beudeker, J. Pen (2002), Stable expression of phytase (phyA) in canola (*Brassica napus*) seeds: Towards a commercial product, *Mol. Breeding*, 10, 31-44.

Ullah AHJ, K. Sethumadhavan, E.J. Mullaney, T. Ziegelhoffer, S. Austin-Phillips (2003), Fungal phyA gene expressed in potato leaves produces active and stable phytase, *Biochem. Biophys. Res. Commun.* 306, 603-609.

Lucca P, R. Hurrell, I. Potrykus (2001), Approaches to improve the bioavailability and level of iron in rice seeds, *J. Sci. Food Agric.* 81, 828-834.

Zimmermann P, G. Zardi, M. Lehmann, C. Zeder, N. Amrhein, E. Frossard, M. Bucher (2003), Engineering the root-soil interface via targeted expression of a synthetic phytase gene in trichoblasts, *Plant Biotechnol. J.* 1, 353-360.

Lucca, R. Hurrell, I. Potrykus (2001), Genetic engineering approaches to improve the bioavailability and the level of iron in rice grains, *Theor. Appl. Genet.* 102, 392-397.

Hamada A, Yamaguchi KI, Ohnishi N, Harada M, Nikumaru S, Honda H (2005), High-level production of yeast (*Schwanniomyces occidentalis*) phytase in transgenic rice plants by combination of signal sequence and codon modification of the phytase gene, *Plant Biotechnol. J.* 3, 43-55.

Hong CY, Cheng KJ, Tseng TH, Wang CS, Liu LF, Yu SM (2004), Production of two highly active bacterial phytases with broad pH optima in germinated transgenic rice seeds, *Transgenic Res.* 13, 29-39.

Brinch-Pedersen H, Olesen A, Rasmussen SK, Holm PB (2000), Generation of transgenic wheat (*Triticum aestivum* L.) for constitutive accumulation of an *Aspergillus* phytase, *Mol. Breeding*, 6, 195-206.

Santosa DA, R. Hendroko, A. Farouk, R. Greiner (2004), A rapid and highly efficient method for transformation of sugarcane callus, *Mol. Biotechnol.* 28, 113-118.

Bali A, Satyanarayana T (2001), Microbial phytases in nutrition and combating phosphorus pollution. *Everyman's Sci* 4, 207-209.

Golovan SP, Hayes MA, Phillips JP, Forsberg CW (2001), Transgenic mice expressing bacterial phytase as a model for phosphorus pollution control. *Nature Biotech* 19, 429-433.

Mohanna C, Nys Y (1999), Changes in zinc and manganese availability in broiler chicks induced by vegetal and microbial phytases. *Anim Feed Sci Technol* 77, 241-253.

Mallin MA (2000), Impacts of industrial animal production on rivers and estuaries. *Am Sci* 88, 26-37.